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TECHNOLOGY

Karolinska Institutet, Stockholm, Sweden

# **HUMAN PAPILLOMAVIRUS AND CERVICAL CANCER: DETECTION OF POTENTIAL MARKERS OF DISEASE PROGRESSION USING LIQUID-BASED CYTOLOGY**

Ingrid Norman



**Karolinska  
Institutet**

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To all women



**Karolinska  
Institutet**

Institutionen för klinisk vetenskap, intervention och teknik.  
Enheten för obstetrik och gynekologi.

# **HUMAN PAPILLOMAVIRUS AND CERVICAL CANCER: DETECTION OF POTENTIAL MARKERS IN DISEASE PROGRESSION USING LIQUID-BASED CYTOLOGY**

## **1 AKADEMISK AVHANDLING**

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## ABSTRACT

This protect aims to evaluate alternative strategies to screen for cervical cancer including liquid-based cytology (LBC) with supplementary reflex testing for human papillomavirus (HPV), p16INK4a, HPV-L1 capsid protein, and miR-205 for use as possible diagnostic and prognostic markers in women with abnormal findings detected through the organized cervical cancer screening program.

This split-sample study enrolled 137 women with atypical Pap smear findings to compare the effectiveness of conventional cytology (CC) with LBC cytology for cervical cancer screening. Sensitivity to detect cervical intraepithelial neoplasia (CIN 2+) was 47% for CC compared with 66% for LBC. These results from these two sampling techniques agreed with histological findings in 37% and 53% of cases, respectively, a statistically significant difference. The significant advantage of improved sensitivity combined with the ability to carry out reflex testing for diagnostic and prognostic markers such as HPV DNA or p16 INK4a without repeated sampling favors LBC for a possible future role as a screening technique.

In cases of low-grade cytological abnormalities combining LBC with HPV triage (LBC+HPV triage) may improve detection of CIN compared with CC. Our study found that CC and LBC+HPV triage produced similar detection rates of CIN2+. When comparing CIN detection rates the adjusted OR for CIN2+ was 0.87 (95% CI: 0.60-1.26) and for CIN3+ 1.00 (95% CI: 0.64-1.58). Consequently, the use of LBC+HPV triage offered no advantage over conventional cytology regarding sensitivity and specificity or positive and negative predictive value for detection of histologically confirmed CIN2+. Nevertheless, LBC+HPV triage may potentially reduce unnecessary medical procedures and testing among HR-HPV-negative women with findings of abnormal cytology.

We analyzed 118 patient samples of dysplastic cells using immunocytochemical staining to assess the expression of p16<sup>INK4a</sup>. Expression levels of p16<sup>INK4a</sup>, correlated with CIN grade, showing stronger reactivity in higher grade lesions. We found a stronger correlation between severity of cytological abnormality and p16<sup>INK4a</sup>, immunoreactivity when the diagnosis was simultaneously confirmed by routine cytology ( $p < 0.001$ ,  $\chi^2$  exact test for trend). This staining technique may be able to serve as a complementary prognostic marker when used as a reflex test to identify women at risk of cervical cancer.

We also used immunocytochemistry to detect L1 capsid protein in HR-HPV-positive women who displayed minor cytological abnormalities. Progression to CIN2+ occurred in 2 of 13 L1-positive women (15.0%) infected with HPV16, compared with 4 L1-positive women infected with other HR-HPV types. Among all L1-positive women with CIN2+, 35.7% were infected with both HR and low-risk (LR) HPV types, 25.0% with HR-HPV types only and 13.3% with HPV16. Loss of previously positive L1 expression may serve as a prognostic marker for preinvasive cervical lesions.

We carried out reverse transcription quantitative PCR (RT-qPCR) on our LBC samples to assess miR-205 expression and correlate these results with histology. Regardless of whether findings were based on histology or cytology, our results showed no significant changes in miR-205 expression relating to different stages of disease progression. It may be that the increased expression of miR-205 is not stage-specific, but could represent a continuous process throughout disease progression, although we cannot conclude this with certainty. Cervical cancer could become one of our most preventable cancers by effectively combining vaccination programs against HR-HPV with improved detection of precursors by integrating molecular markers into screening programs.

# LIST OF SCIENTIFIC PAPERS

This thesis is based on the following Papers:

- I. Zhu Jiu\*, **Norman Ingrid\***, Elfgren Kristina, Gaberi Vera, Hagmar Bjorn, Hjerpe Anders, Andersson Sonia. (\*Equal contribution)  
**A comparison of liquid-based cytology and Pap smear as a screening method for cervical cancer.** Oncol Rep. 2007 Jul;18(1):157-60.  
PMID:17549362
- II. Fröberg Maria, **Norman Ingrid**, Johansson Bo, Hjerpe Anders, Weiderpass Elisabete, Andersson Sonia.  
**Liquid-based cytology with HPV triage of low-grade cytological abnormalities versus conventional cytology in cervical cancer screening.** Curr Pharm Des. 2013;19(8):1406-11. PMID:23016773
- III. **Norman Ingrid\***, Brismar Sophia\*, Zhu Jiu, Gaberi Vera, Hagmar Bjorn, Hjerpe Anders, Andersson Sonia. (\*Equal contribution)  
**p16(INK4a) immunocytochemistry in liquid-based cervical cytology: is it feasible for clinical use?** Int J Oncol. 2007 Dec;31(6):1339-43.  
PMID:17982660
- IV. **Norman Ingrid**, Hjerpe Anders, Andersson Sonia.  
**High-risk HPV L1 capsid protein as a marker of cervical intraepithelial neoplasia in high-risk HPV-positive women with minor cytological abnormalities.** Oncol Rep. 2013 Aug;30(2):695-700. doi: 10.3892/or.2013.2538. Epub 2013 Jun 11. PMID:23756570
- V. Xie Hong, **Norman Ingrid**, Hjerpe Anders, Larsson Catharina, Lui Weng-onn, Andersson Sonia.  
**Expression of microRNA in liquid-based cytology, a possible prognostic marker for cervical cancer.**  
In manuscript.

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## LIST OF ABBREVIATIONS

ADCA	Adenocarcinoma
AGUS	Atypical glandular cells of undetermined significance
AGC-H	Atypical glandular cells-favor high-grade lesion
AIS	Adenocarcinoma in situ
ASC-H	Atypical squamous cells-favor high-grade lesion
ASCUS	Atypical squamous cells of undetermined significance
CC	Conventional Cytology
CIN	Cervical intraepithelial neoplasia
CIN2+	Histological confirmed CIN2 or more advanced lesion
CIS	Carcinoma in situ
DNA	Deoxyribonucleic acid
E	Early
HPV	Human papillomavirus
HR-HPV	High-risk HPV
HSIL	High-grade squamous intraepithelial lesion
HTX	Hematoxylin
ICC	Invasive cervical cancer
L	Late
LBC	Liquid-based cytology
LBC-HPV testing	Liquid-based cytology screening with supplementary HPV
LPP	Low predictive power
LR-HPV	Low-risk HPV
microRNA	Small non-coding RNAs
LSIL	Low-grade squamous intraepithelial lesion
p16 <sup>INK4a</sup>	Protein 16 (inhibits cyclin-dependent kinases 4)
p53	Protein 53
Pap	Papanicolaou
pHR-HPV	Probable HR-HPV
PCR	Polymerase chain reaction
pRb	Retinoblastoma protein

PV	Predictive value
RNA	Ribonucleic acid
SCC	Squamous cervical cancer
TBS	The Bethesda system
TP	ThinPrep
TZ	Transformation zone
WHO	World Health Organization
WNL	Within normal limits
N/C ratio	nuclear/cytoplasmic ratio



## 1. POPULÄRVETENSKAPLIG SAMMANFATTNING

Cervixcancer (cxca) (livmoderhalscancer) är i världen en av de vanligaste cancerformerna hos kvinnan och kan med dagens teknologi förebyggas. Detta har i Sverige varit målsättning att med hjälp av den organiserade cellprovskontrollen kunna sänka mortaliteten. Det förefaller som vi med stor framgång sedan 1960-talets mitt har lyckats sänka cancer frekvensen med mer än hälften trots det enstaka provets begränsade sensitivitet på 50-70 %. Begränsade faktorer för programmets effektivitet är framför allt graden av hörsamhet och det cytologiska provets känslighet. Detta kompenseras genom tämligen korta intervall för provtagning. Med ett sådant intervall på 3 år testas kvinnan i genomsnitt med ca 12 prover under sin livstid, förutsatt att kvinnan hörsammat alla kallelser. Denna provtagnings frekvens ger en mycket hög känslighet för att i något av dessa prover kunna diagnostisera en förändring som behandlingsbar. En av förutsättningarna för den framgångsrika cellprovskontrollen har varit att den cytologiska diagnostiken utförts med tillräcklig noggrannhet samtidigt som man gör ansträngningar för att undvika överdiagnostik.

De svåraste cytologiska bedömningarna är gränsvärden mellan tidiga cellförändringar och reaktiva förändringar. Det rekommenderas i Sverige att diagnostiken ska drivas så att ca 5 % av cellproverna rapporteras som avvikande. Denna siffra skall ses mot bakgrund av den totala livstidsrisken att utveckla cervixcancer, som är under 1 %. Låg specificitet för dessa svårbedömda förändringar som inte har med cancerutvecklingen att göra. Dessa uppföljande undersökningar skapar dels onödig oro hos en stor grupp friska kvinnor och bidrar dessutom till betydande kostnader för samhället. Konventionell gynekologisk provtagningsteknik med cellutstryk på objektglas har, trots framgångsrik tillämpning, en begränsad möjlighet till kompletterade analyser. Kvinnan måste kallas tillbaka för ytterligare en provtagning vilket skapar onödig oro och begränsar samtidigt provets reproducerbarhet och sensitivitet. Det konventionella provet har på grund av sin hantering en stor risk för artefakter med lufttorkade celler, som försvårar diagnostiken och därmed minskar provets specificitet.

Alternativa provtagningstekniker för cellprover har utarbetats, och dessa förbättrar betydligt provets kvalitet. Den vätskebaserade tekniken innebär att provtagaren direkt

slammar upp provet i en buffrad fixeringslösning, (vätskebaserad cytologi, liquid-based cytology, LBC), i stället för att cellerna stryks ut på ett objektglas. LBC-provet behålls sedan i detta fixativ tills man på laboratoriet överför cellerna till objektglas. Tekniken ger morfologiskt bättre bevarade celler och när denna teknik testas på olika laboratorier har detta ibland, men inte alltid, höjt provets sensitivitet. Vid den cytologiska bedömningen av dessa preparat används bara en mindre del av provet till rutindiagnostik, dvs. en betydande del av provet finns kvar för kompletterande analyser som gör det möjligt att öka provets specificitet. En sådan hjälpanalys kan vara påvisande av högrisktyper av humant papillomavirus (HR-HPV). Med LBC kan detta utföras utan att kvinnan behöver kallas till kompletterade undersökningar (s.k. ”reflex-screening”), och om de förändrade cellerna inte innehåller HR-HPV kan onödig uppföljning och oro undvikas.

I dag vet vi att i princip all cxca orsakas av en persisterande infektion med HR-HPV. HR-HPV är en nödvändig men inte en tillräcklig förutsättning för en cancerutveckling i cervix. Virusets skadar cellens genetiska stabilitet och är närvarande under hela carcinogenesen. E6 och E7 generna i HR-HPV vironen driver utvecklingen av precancerösa cellförändringar genom att blockera tumörsuppressor proteinerna p53 och pRb, vilket avsevärt ökar risken för genetiska skador och klonal utmognad. I avancerade förändringar integreras HPV genomtet med värdcellens kromosomer vilket ökar förmågan hos E6 och E7 att initiera en neoplastisk transformation.

Flertalet infektioner med HR-HPV läker spontant, och infektionen är inte den enda faktorn som leder till utveckling av cancer. Om sådana riskfaktorer kan identifieras skulle det öka förståelsen för den HPV-relaterade cancerutvecklingen. Eventuellt skulle detta också ge möjlighet att med specifikt identifiera de kvinnor som löper risk att utveckla cancer. De senaste årens framsteg inom molekylärbiologi och genteknologi öppnar helt nya möjligheter till en ytterligare optimerad diagnostik för att kunna öka det diagnostiska utbytet av materialet. Dessa tekniker kan användas vid undersökningar av det vätskebaserade provet.

Denna studie syftar till att studera hur man med denna provtagningsmetodik kan kombinera informationen från cytologiska, virologiska och molekylärbiologiska

analyser från ett och samma prov, för att mer specifikt hitta de kvinnor som löper risk att utveckla cervixcancer.

Det första arbete ville kartlägga i vad mån LBC kan ge högre känslighet att hitta en höggradig cellförändring i jämfört med konventionellt tagna prover på ett laboratorium med lång cytologisk erfarenhet. Resultatet visade att en ökad känslighet för LBC i kombination med möjligheten till reflex-analys såsom, HPV DNA eller p16<sup>INK4a</sup> immuncytokemi utan förnyad provtagning, gör LBC teknologin till en viktig provtagningsmetod med betydande fördelar.

Med den andra studien ville vi i större skala utvärdera de båda teknikerna för provtagning. Känsligheten för LBC teknologins utförande minskade när tekniken implementerades inom det populationsbaserade screeningsprogrammet. Studien reflekterade även upplärningsprocessen som förbättrades med tiden efter att tekniken blev implementerad.

I det tredje delarbetet studerade vi om upplagring av p16<sup>INK4a</sup> protein ("cancermarkör"), i immuncytokemisk metod, kan närvara vid en HPV-orsakad cellförändring. Studien visade goda resultat där p16<sup>INK4a</sup> upplagring i cellkärnan ej påvisades i några normala celler utan enbart i avancerade cellförändringar, histologiskt verifierade. Detta gör att denna "biomarkör" kan användas för att öka känsligheten att hitta höggradiga cellförändringar i ett cellprov som vidare behöver följas upp.

Syftet med den fjärde studien var att studera HPV L1 capsid protein som kan ha en självläkande effekt hos kvinnor med en HPV infektion. Studien visade att uttryck av L1 proteinet i cellkärnan var mer associerad till lättare cellförändringar än till höggradiga cellförändringar och att ett L1 uttryck kan initiera en spontanläkning. Tillsammans med p16<sup>INK4a</sup> kan HPV L1 förbättra diagnostiken för att mer specifikt hitta de kvinnor som löper risk att utveckla livmoderhalscancer.

I den femte studien testade vi hypotesen att nivåer av ett specifikt microRNA uttryck förändras i takt med cancerutvecklingen. Resultatet visade att få tumörceller i

förhållande till normala celler och närvaro av tumörnekros, gör LBC mindre lämplig som metod för denna analys.



## 2. INTRODUCTION

Cervical cancer (cxca) is one of the most common cancers among women worldwide. The goal in Sweden has been to reduce mortality through a population-based screening program, which since 1965 has cut mortality from cxca by more than half despite limited sensitivity of about 70% for a single Pap smear. This limited sensitivity is partially compensated by relatively short sampling intervals. A successful screening program requires sufficient predictive value (PV) for the diagnosis. Low predictive power (LPP) for minor cytological abnormalities results in a large number of follow-up cases for which risk for developing cancer is low, thereby causing unnecessary concern for a large group of healthy women and resulting in substantial costs to society.

Conventional gynecological sampling with the Papanicolaou (Pap) smear offers limited opportunity for supplementary analyses. Samples taken using liquid-based cytology (LBC) techniques preserve cellular morphology better, which has the advantage of increasing sensitivity and the material can also be used for reflex-testing to improve specificity. LBC technology with reflex-testing for high-risk Human papillomavirus (HR-HPV) DNA has now become an established method for improving test performance.

Today we know that virtually all cxca is caused by persistent HR-HPV infection. High-risk HPV infection is a necessary, albeit insufficient, risk factor on its own for causing cxca. The virus interferes with cellular genetic stability and is present throughout the entire process of carcinogenesis. The E6 and E7 HPV oncogenes inhibit the effect of tumor suppressor proteins, protein 53 (p53) and Retinoblastoma protein (pRb), thereby increasing the risk of mutations. Most HR-HPV infections are transient. Additional as yet unknown factors are necessary for development of cxca. Identification of such risk factors would improve understanding of HPV-related cancer development.

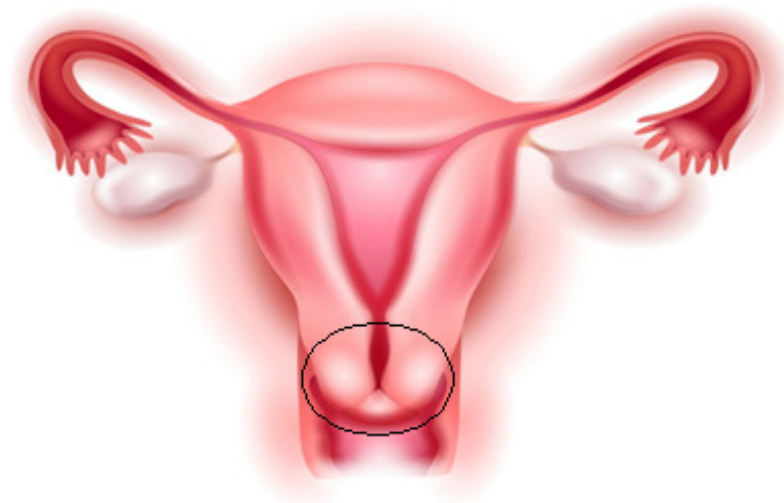
The aim of this project was to evaluate what additional diagnostic protection LBC offers in cytology screening and to search for diagnostic and prognostic markers in LBC samples. The HPV L1 capsid protein, p16INK4a, and microRNA are two such interesting candidate markers that can be analyzed in LBC samples. Effective use of

complementary analyses such as these will help to more specifically identify women at risk for developing cxa.

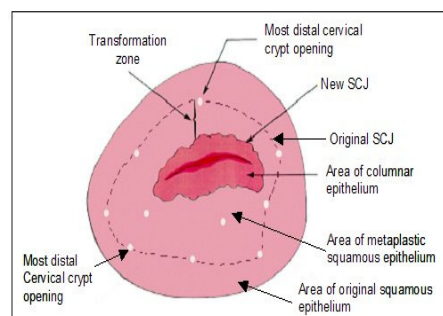
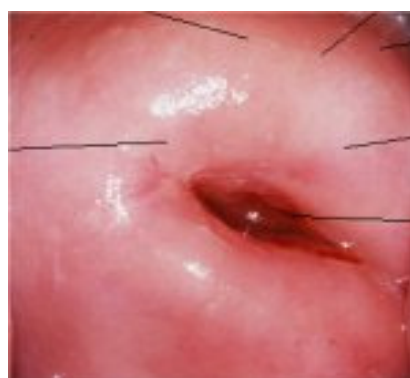
### 3. BACKGROUND

#### 3.1. THE UTERINE CERVIX

The uterine cervix is the distal end of the uterus that protrudes into the upper vagina. The cervix is about two-three cm long and about two cm in diameter (Figure 1). The vaginal part of the cervix, the ectocervix, is primarily covered with non-keratinized stratified squamous epithelium which potentially may become keratinized. Cell division is normally confined to the basal cells of the basement membrane. As the parabasal cells detach from this membrane they begin to differentiate, eventually forming the intermediate and superficial cell layers. This maturation process take 6-12 days in fertile women (Doorbar, 2013).The endocervical canal (endocervix) is lined with mucus-secreting columnar cells.



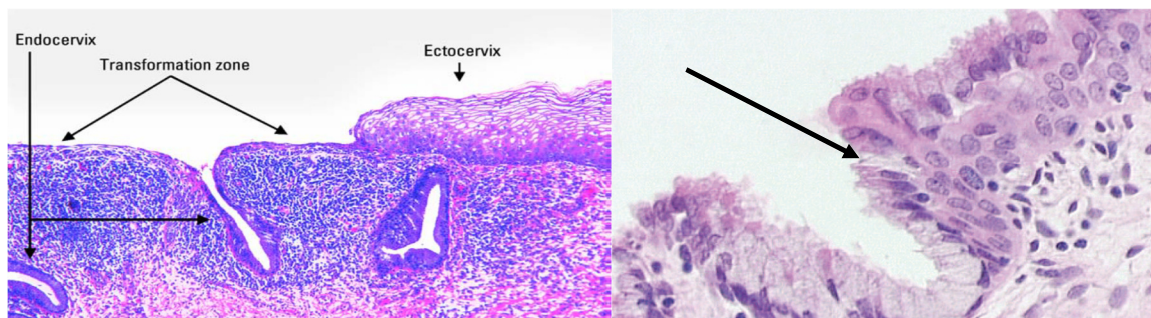
**Figure 1.** The female internal organs. The uterine cervix incircled. Colposcopy, cytology and histopathology pictures. With permission from : <http://screening.iarc.fr>



**FIGURE 5.1:** A method of identifying outer and inner borders of the transformation zone (SCJ: Squamocolumnar junction)

**Figure 2.** The uterine cervix and transformation zone. Colposcopy, cytology and histopathology pictures. With permission from: <http://screening.iarc.fr>

The boundary between squamous and columnar epithelium is called the squamocolumnar junction (Figure 2). Before puberty and after menopause this junction is not found on the ectocervix. Instead, in post-menopausal women the epithelium becomes atrophic, causing the junction to migrate up into the cervical canal. In fertile women squamous metaplasia is common in this junction (Schiffman, Castle, Jeronimo, Rodriguez, & Wacholder, 2007), forming the transformation zone (TZ). The metaplastic epithelium of the TZ is thin (Figure 3), which makes the basal cells anchored to the basement membrane sensitive to infectious agents with a potential for malignant transformation (Doorbar et al., 2012; Sellors J.W., 2003).

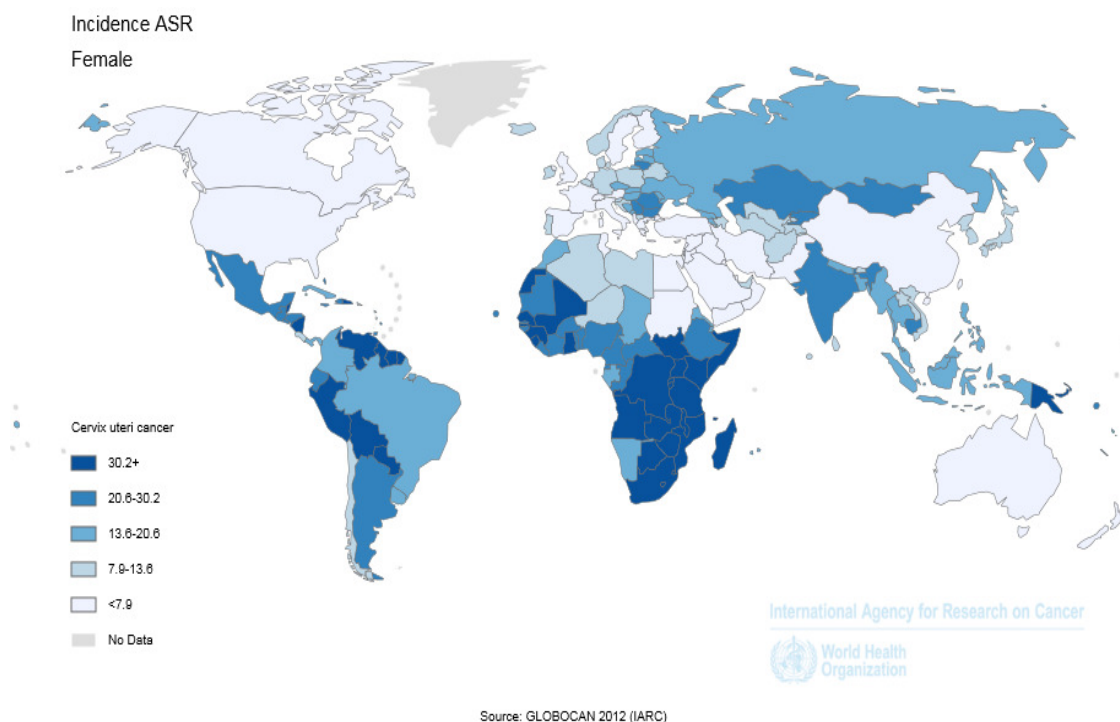


**Figure 3.** The cervical transformation zone. With permission from Mark Schiffman, Bethesda USA.

### 3.2. CERVICAL CANCER

Cervical cancer (cxca) is a current global health problem. This preventable disease accounts for almost 12% of all female cancers worldwide and is the fourth most common cause of cancer mortality among women. Worldwide there are almost 528,000 new cases of this disease per year, resulting in more than 265,000 deaths annually (Ferlay et al., 2015). The incidence is considerably higher in developing countries, where more than 87% of cases can be found. Thus incidence varies geographically and in some regions this disease is the most common type of cancer (Figure 4). The rate of cxca is especially high, over 30 per 100,000, in East Africa (42.7) and Melanesia (33.3), as well as Southern (31.5) and Middle (30.6) Africa. The average global incidence is 14/100,000 with a mortality rate of 6.8/100,000.

In Sweden, the corresponding figures are 7.4/100,000 and 1.9/100,000, respectively, while in countries such as Tanzania these figures are as high as 40.6/100,000 and 32.5/100,000 annually (Ferlay, et al., 2015). The lowest incidence rates can be found in Western Asia (4.4/100,000) and Australia/New Zealand (5.5/100,000). European countries such as Switzerland, Malta, Cyprus and Finland have the lowest incidence with about 4/100,000 annually, while Romania, Lithuania and Bulgaria have much higher rates with over 24/100,000 women-years (Ferlay, et al., 2015).



**Figure 4.** Incidence data per 10.000. Ferlay J, Soerjomataram I, Ervik M, Dikshit R, Eser S, Mathers C, Rebelo M, Parkin DM, Forman D, Bray, F. GLOBOCAN 2012, Cancer Incidence Worldwide: IARC CancerBase No. 11 Available from: <http://globocan.iarc.fr>, accessed on day/month/year.

In Sweden the 2013 incidence translates to 468 cases of cancer and 180 cancer deaths, a survival rate of 73% (socialstyrelsen.se, 2013). The worse prognosis seen in developing countries is associated with limited availability of hospital resources and women waiting too long to seek medical care. This disease was first described by Rigoni-Stern in 1842 (Rigoni-Stern, 1842) who also observed that cxca is related to

sexual activity and reproduction. Many risk factors for cervical cancer have been demonstrated, including early sexual debut, higher number of sexual partners, smoking and long-term use of oral contraceptives (Louie et al., 2009; Shavit, Roura, Barchana, Diaz, & Bornstein, 2013). A low incidence can be found among nuns (Fraumeni, Lloyd, Smith, & Wagoner, 1969) and women without a sexual partner (Fraumeni, et al., 1969). Studies have also shown that male circumcision reduces the incidence (Gray et al.) even when multiple partners are involved (Castellsague et al., 2006). Thus, development of cxc is strongly related to environmental factors and lifestyle, early sexual debut and multiple partners as key risk factors.

### **3.3. HISTOLOGICAL CLASSIFICATION**

In 1968, a new histological classification of precursor lesions was developed, designated Cervical Intraepithelial Neoplasia (CIN). The term CIN implies a neoplastic lesion without specifying degree of severity (Barron & Richart, 1968; Fu, Reagan, & Richart, 1981). The classification includes evaluation of epithelial maturation. Thus in CIN1 (Grade 1), the least severe degree of cellular change, the border between the parabasal and intermediate squamous cells is still visible in the basal third of the epithelium. Such lesions are often difficult to differentiate from reactive changes. In CIN2, atypical parabasal cells are found in the middle third of the epithelium, whereas in CIN3, equivalent to carcinoma-in-situ (CIS), they occupy more than two-thirds of the total epithelial thickness. Histopathology serves as the gold standard for quality control of cytology and colposcopy.

### **3.4 CYTOLOGICAL CLASSIFICATION**

In 1988, a working group at the National Cancer Institute (Bethesda, USA) introduced the Bethesda system for cervical/vaginal cytological diagnoses (Solomon et al., 2002). The goal was to provide uniform diagnostic terminology to facilitate communication between laboratory and clinician. This Bethesda system allowed for modification of classification if necessary and was duly revised in 2001 in response to new technologies and findings. The Europe Against Cancer Program introduced European guidelines for

quality assurance in cervical cancer screening The recommendation was for cytology laboratories to apply a national cytology terminology with uniform grading of cellular abnormalities, in parallel with the Bethesda reporting system.

The current Swedish classification system can be translated into classification according to the 2001 Bethesda system. However, one difference is that samples with koilocytosis without cellular atypia are reported as within normal limits (WNL) in Sweden. Low-grade lesions include atypical squamous cells of undetermined significance (ASCUS) and CIN1. Squamous cell changes classified as high-grade squamous intraepithelial lesions (HSIL) are graded as CIN2 or CIN3. Cell changes that raise suspicion of an HSIL lesion are grouped as ASC-H (atypical squamous cells, favor high-grade lesion). Adenocarcinoma in situ (AIS) and adenocarcinoma (ADCA) represent about 20% of all cervical cancers (Gien, Beauchemin, & Thomas, 2010). The Bethesda system discriminates between atypical glandular cells of undetermined significance (AGUS), atypical glandular cells, favor neoplastic, AIS and ADCA (Solomon, et al., 2002).

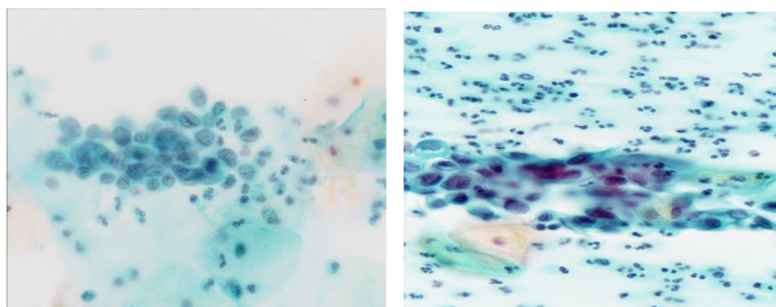
The information of sample adequacy as an important part of the cytology report has been a contributions of TBS. The guidelines constitute the adequacy of the sample for the detection of abnormalities of the uterine cervix: (i) patient and sample identification, (ii) relevant clinical information, (iii) adequate number of well-preserved epithelial cells (iiii) cellular composition and sampling of the transformation zone. The Bethesda system defines a fully satisfactory sample as containing both squamous cells and endocervical or squamous metaplastic cells. These cellular component form the microscopic basis that the TZ has been sampled (Kurman, Henson, Herbst, Noller, & Schiffman, 1994).

### **3.4.1. SAMPLING**

Pap smear cytology, which is recommended by the WHO and still in use, involves collecting exfoliated cells from the ectocervix and endocervix, and in some areas from the fornix. The best sampling tools are a combination of a wooden spatula (Ayre or Aylesbury) and an endocervical brush, (Cytobrush) (Arbyn et al., 2007), but other

sampling devices are also available, including a broom-like device (Bigras et al., 2003). The cells from each tool are smeared on separate glass slides and immediately fixed in 95% ethanol to prevent air-drying artifacts, which should be carefully avoided since they affect staining and the texture of nuclei and cytoplasm. After fixation the slides can be dried without risk of inducing artifacts that interfere with subsequent Papanicolaou staining.

Liquid-based cytology (LBC) is an alternative method that was developed to improve sampling (Figure 5). Today, this method is becoming common, especially in many countries with a high economic standard. Two general techniques are available: ThinPrep® (TP) (Hologic, Marlborough, MA, USA) and SurePath™ (BD, Franklin Lakes, NJ, USA). The US Food and Drug Administration (FDA) approved SurePath™ in 2004 and the TP test in 1996, based on split-sample analysis (Limaye, Connor, Huang, & Luff, 2003).



Pap smear

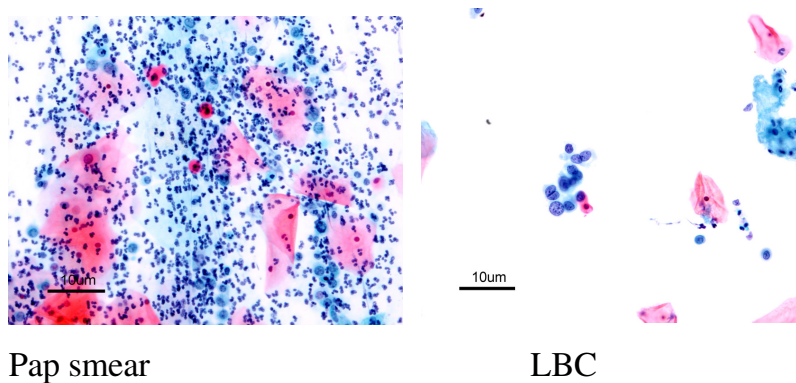
LBC

**Figure 5.** Squamous cells with high grade lesions seen in the conventional Pap smear and in an LBC sample

LBC technique involves obtaining samples using either a broom-like device or the combination of an endocervical brush and plastic spatula, after which the cells are directly stirred into a vial containing buffered fixative solution. The LBC sample is stored in this fixative until further processing in the laboratory. LBC enables more rapid fixation. The fixative contains hemolytic and proteolytic agents that separate epithelial cells from blood and mucus during filtration. In the TP technique, the cells are then collected on a filter membrane, after which they are transferred to a glass slide using an



automated TP Processor. This sample preparation method produces an almost single layer of cells without drying artifacts.



**Figure 6.** Squamous cells are easily demonstrated in the LBC sample, while they in the Pap-smear are hidden under a curtain of inflammatory cells

This technique is superior for preserving morphology (Figure 6), while making the remaining cell suspension available for supplementary analyses to improve test performance. (Obwegeser & Brack, 2001; Park, Jung, Kim, & Choi, 2007; Ronco et al., 2007; Schledermann, Ejersbo, & Hoelund, 2006). The thin uniform layer of well-preserved cells facilitates microscopic interpretation. This method can be used to improve screening program performance. (Strander, Andersson-Ellstrom, Milsom, Radberg, & Ryd, 2007; Tsonev, Ivanov, Kovachev, Kornovski, & Ismail, 2013; Zhu et al., 2007). Other studies have failed to demonstrate any significant difference between LBC and conventional cytology (Arbyn et al., 2008; Froberg et al., 2013; Siebers et al., 2009; Tsonev, et al., 2013). At the time the second study was being conducted, LBC and HPV testing were new laboratory techniques, for which reason the learning curve may have influenced the results. Thus one advantage of LBC is that a significant portion of the sample remains after processing, which allows for adjuvant analysis to increase specificity. Such adjuvant analyses may include testing for high-risk human papillomavirus (HR-HPV), or immunocytochemistry to identify possible progression and diagnostic markers, both of which may facilitate distinguishing premalignant lesions from reactive changes (Bergeron et al., 2014).

### 3.4.2. STAINING

The staining protocol for both conventional and LBC samples is polychromatic using one nuclear staining dye, acidified Harris Hematoxylin (HTX), and two counterstains, Orange G-6 (OG) and Eosin Azure-50 (EA) (G. N. Papanicolaou, 1942). To obtain a better chromatin pattern HTX stains the nucleus a crisp blue. This dye binds to chromatin-associated proteins (histones and other structures), rather than to the nucleic acid itself (Frost, 1997). OG-6 counterstain is an acidic dye that stains superficial squamous cells an intense orange to yellow color, as it binds to keratin and glycogen. EA-50 is in itself a polychromatic staining solution, containing light green SF and eosin Y that stain several cellular components. This dye therefore colors the cytoplasm of intermediate and parabasal cells, resulting in both a pink and a turquoise-green-to-blue appearance (Boon, 1996; Keebler, 1997)

## 3.5. CYTOMORPHOLOGY. CRITERIA FOR EPITHELIAL CELL ABNORMALITIES

**3.5.1. ASCUS** means that it cannot be decided if the lesion is a precancerous one, hence the term “undetermined significance”. The nuclear enlargement is two and a half to three times that of a normal intermediate squamous cell. Variation in nuclear size, shape and binucleation may be observed. Mild hyperchromasia may be present, but evenly distributed without granularity. Nuclear outlines usually smooth and regular.

**3.5.2. Low-grade squamous lesion (*LSIL*)** is classified as cellular changes associated with koilocytosis, with or without simultaneous mild dysplasia/CIN1. Nuclear abnormalities are generally confined to cells with intermediate or superficial cell cytoplasm. The nuclear are enlarged at least three times compare to normal intermediate nuclei, with correspondingly increased nuclear/cytoplasmic (N/C) ratio. The chromatin is evenly distributed but slightly coarse and the nuclear membranes are slightly irregular. A moderate variation in nuclear size is often present. Nucleoli are rarely presents. To avoid confusion with molecular tests for HPV, it is required in Sweden that koilocytosis without nuclear atypia is reported as WNL.

**3.5.3. High-grade squamous lesion (HSIL)** includes changes associated with moderate dysplasia/CIN2 and severe dysplasia CIN3/CIS. The cells usually show predominant nuclear abnormalities, including nuclear irregularities, folding and hyperchromasia with coarsely granular chromatin without macronucleoli. The nuclei are not larger than in LSIL but the cytoplasm is decreased leading to increased N/C ratio. The main criteria separating HSIL into CIN2 or CIN3/CIS are the N/C ratio and tendency to dissociate. In CIN3/SIC lesion the abnormal cells also occur singly, often in the form of more or less naked nuclei (“grapes”).

**3.5.4. In non-keratinizing squamous cell carcinoma (SCC)** the cells occur singly or in syncytial-like aggregates. They have all the features of HSIL together with prominent macronucleoli and irregular distribution of chromatin. The SCC often has an associated tumor diathesis with necrotic debris and old blood.

**3.5.5. In keratinizing SCC** the cells occurs more often singly and less commonly in aggregates. There is a more prominent polymorphism with cells showing orangeophilic cytoplasm. Nuclei vary markedly in size, macronucleoli being less common than in the non-keratinizing tumor.

**3.5.6. Adenocarcinoma in situ (AIS)** *presents with* groups and strips of cells, rosette formation, feathering, crowding and/or pseudostratification. The cells show increased N/C ratio with fine to coarsely granular chromatin, evenly distributed and most often hyperchromatic with prominent nucleoli.

Cells from a ***cervical adenocarcinoma*** occur in 2- and 3-dimensional clusters and sheets, sometimes with finely vacuolated cytoplasm. Round to oval enlarged nuclei with fine to coarsely granular patterns. There is parachromatin clearing and irregular nucleoli are often prominent and multiple.

The major microscopic differences between the Pap smear and the LBC sample are the cellular distribution and cell preservation. The Pap smear has thick and thin areas with artifacts after the mechanical distortion of the cell material. In LBC samples, the cells

are well preserved due to immediate fixation and the preparation is uniform with evenly distributed cells. The LBC sample is almost monolayer with less overlapping cells. The nuclear hyperchromasia associated with squamous lesions in the Pap smear may be less extensive in LBC samples, but the nuclear morphology is enhanced, primarily as the result of the immediate LBC fixation. Another feature of the fixation, the cell in LBC samples tend to round up and be smaller in liquid-based preparation.

Many of the low grade lesions seen in LBC samples cannot be recognized in conventional smears, explaining why LBC can be conducted with high sensitivity, improving the sensitivity and preserving the specificity by reflex analysis of HPV DNA. Nuclei may, however, be over-interpreted and it is important to carefully evaluate the nucleus under high power, especially in the absence of hyperchromasia.

In general, the LBC cell pattern is similar to what is observed on well-preserved Pap smear. However, unlike the Pap smear, the background in LBC is generally clean and single cells are more prominent. The presence of blood, inflammation and diathesis is less apparent compared to the conventional Pap smear. The ability to recognize the tumor diathesis is essential when considering a malignant process in the LBC samples.

### **3.6. HUMAN PAPILLOMAVIRUS**

#### **3.6.1. HPV classification**

Papillomaviruses (PVs) belong to the Papovaviridae family. Almost 200 different HPV types, belonging to 49 species, have been characterized ([WWW.hpvcenter.se](http://WWW.hpvcenter.se), accessed on 2014-11-25) and modern sequencing techniques will continue to add new types (Arroyo Muhr et al., 2014; Bzhalava, Eklund, & Dillner, 2015; Eklund, Forslund, Wallin, & Dillner, 2014). HPV virus is highly species-specific, does not change host, and has been genomically stable for millions of years.

PV nomenclature is established by the International Committee on Taxonomy of Viruses (ICTV) based on the Study Group of Papillomavirus (Bernard et al., 2010; Z. Chen, de Freitas, & Burk, 2015; de Villiers, Fauquet, Broker, Bernard, & zur Hausen, 2004). Different PVs infect mammals and birds. The viruses are classified according to

taxonomic levels: genus, species, type, subtype and variant (Bzhalava, et al., 2015). PV form five major genera: *Alpha*-papillomavirus, *Beta*-papillomavirus, *Gamma*-papillomavirus, *Mu*-papillomavirus and *Nu*-papillomavirus. Different *genera* have less than 60% similarity within the L1 genome. *Species* within a genus share about 60-70% nucleotide similarity and newly isolated HPV *types* must be at least 10% different from any other known HPV type. Subtypes differ by 2-10% and variants by <2% when compared with the most similar known HPV type (Bernard, et al., 2010).

Alpha-papillomaviruses are divided into two groups: cutaneous and mucosal. The genital mucosal alpha genus has been studied most because it contains the viruses that cause cxa (Doorbar, 2013). Today, novel HPV types are assigned a number after the whole genome has been cloned and sent to the International HPV Reference Center (Bernard, et al., 2010; de Villiers, et al., 2004). Currently about 40 different HPV types have been found to infect genital epithelium. Mucosal types are subdivided into high-risk (HR) and low-risk (LR), based on oncogenic potential (Bernard, et al., 2010). LR-HPVs, such as types 6 and 11, are mainly associated with benign genital warts, while HR-HPVs are carcinogenic agents for cxa.

In 2012, the International Agency for Research on Cancer (IARC) concluded that there was consistent and sufficient epidemiological, experimental and mechanistic evidence for carcinogenicity in 12 HR-HPV types (HPV16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, and HPV59) ("Biological agents. Volume 100 B. A review of human carcinogens," 2012). All of them belong to the  $\alpha$ -Papillomavirus family and are considered to be 1A carcinogens. Lesser evidence is available for a thirteenth agent, HPV68, which is classified as a 2A carcinogen. In one recent study, 96% of evaluated cancer cases could be attributed to one of the 13 HR-HPV types.

In light of additional data (Halec et al., 2014; Halec et al., 2013), evidence indicates that an upgrading of HR-HPV types could be considered. To specify numbers to new HPV types, an international HPV center was established at the German Cancer Research Center in 1985 (Bernard, et al., 2010; de Villiers, 2013). This International HPV

Reference Center stores reference clones and distributes samples to researchers. In 2012, this center was transferred to Karolinska Institutet at Huddinge.

### **3.6.2. HPV genome**

HPVs are small double-stranded circular DNA viruses with about 8000 base pairs. The genome of HPV16, the most studied type, is organized into three different regions: (i) early (E) genes (E1, E2, E4, E5, E6 and E7), (ii) a region containing two late (L) genes that encode the major L1 and the minor L2 capsid proteins, and (iii) the long control region (LCR), located between L1 and E6 (Bernard, et al., 2010).

HR-HPV E6 and E7 are viral oncogenes that play a key role in carcinogenesis by transforming and immortalizing cells. E6 is able to inactivate the p53 tumor suppressor protein, thereby interfering with DNA repair and apoptosis. The primary target of E7 is the pRb tumor suppressor protein that both controls transcription factor E2F and exerts negative

regulatory control on the G0/G1 phase of the cell cycle. E7 may induce abnormal cell proliferation by inactivation of pRb, which ultimately results in failure to prevent cells with

damaged DNA from entering into s-phase and undergoing subsequent mitosis.

This process creates a less stable genome with increased risk of mutations (Doorbar, et al., 2012; M. Stanley, 2010; M. A. Stanley, 2012) (Moody & Laimins, 2010; Tommasino, 2014).

<b>E1:</b>	Mainly controls viral DNA replication
<b>E2:</b>	Responsible for gene transcription and viral replication
<b>E4:</b>	Mediates virion particle release by destabilizing keratin in the cytoskeleton
<b>E5:</b>	Stimulates growth factors through inhibition of apoptosis
<b>E6:</b>	Inactivates p53-dependent apoptosis
<b>E7:</b>	Inactivates members of the pRb family of tumor suppressor proteins by promoting cell cycle progression
<b>L1:</b>	Assembly of capsomeres from viral genome
<b>L2:</b>	Works together with L1 on assembly of viral capsid; participates in viral entry process of infection
<b>(Bosch et al., 2013; Moody &amp; Laimins, 2010)</b>	

**Figure 7.** The HPV genome of HPV16. Functions in early (E) and late (L) regions.

### 3.6.3. The HPV infection

Genital warts have been known since antiquity. Even in ancient Rome the link between sexual activity and the presence of such warts was noted. Genital wart infection as a consequence of sexually transmitted infection was only confirmed in the twentieth century. In 1907, Ciuffo (Ciuffo, 1907) showed that human skin warts contained an infectious substance, identified as a submicroscopic organism. In 1935, Rous and Beard demonstrated that the virus could induce papillomas in rabbits (Rous & Beard, 1935), which was eventually confirmed by Stauss et al., 1949, who visualized the viral particles in wart extracts, using electron microscopy (Strauss, Shaw, & et al., 1949).

The suspicion that cxa can be caused by an infectious agent was first raised in the 1840s (Rigoni-Stern, 1842). In the late 1960s, this form of cancer was associated at first with Herpes Simplex Virus 2 (HSV2) (Goldberg & Gravell, 1976). However, in the 1970s, this hypothesis was abandoned (Lehtinen et al., 1992; Lehtinen et al., 1989), and

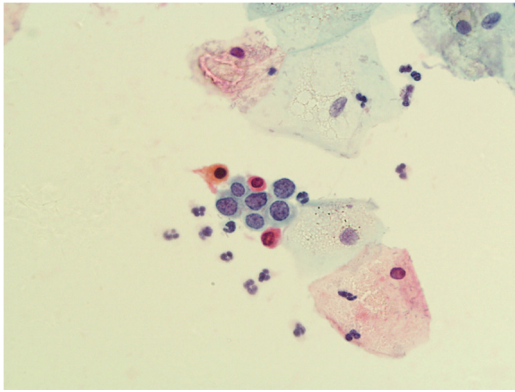
the focus shifted to findings which showed that HPV played a central role in the development of cxa (J. zur Hausen, Schulte-Holthausen, Wolf, Dorries, & Egger, 1974)). The causative role of HR-HPV for the development of cxa was subsequently established (H. zur Hausen, 1977, 1998, 2009). In 1983 HPV16 was identified in cxa biopsies (Durst, Gissmann, Ikenberg, & zur Hausen, 1983), and by the next decade both HPV 16 and HPV 17 had been cloned. These two HPV types are found in approximately 70% of cxa cases worldwide (J. S. Smith et al., 2007). In 2008 Harald zur Hausen was awarded the Nobel Prize in Physiology or Medicine, for his role in the discovery that HPV is the causal agent of cancers.

HPV infection is one of the most common sexually transmitted infections in the world (M. Stanley, 2010). Nearly 100% of all cervical cancers contain HR-HPV DNA (Bosch et al., 1995; Munoz et al., 2003; J. S. Smith, et al., 2007). Persistent infection with HPV is a prerequisite in almost every case of cervical cancer, but is insufficient as a sole agent to cause cervical cancer (Walboomers et al., 1999). HPV infection increases the risk for developing cancer in a multistep process (Schiffman, et al., 2007).

Lifetime risk of HPV infection has been estimated at about 80% (Koutsky, 1997) depending on various cultural factors. Time from infection to release of complete viral particles is about two-three weeks (M. A. Stanley, 2012). However, the majority of HR-HPV infections are transient and clear spontaneously within six to twelve months (Doorbar, 2006; H. zur Hausen, 2009). Global HPV prevalence is about 10%, albeit with great variation. Prevalence is low in Eastern Asia (6%), but high in Eastern Africa (31%). In Northern Europe it is estimated at about 7%, with large differences among age groups ((Ferlay, et al., 2015; Wahlstrom, Iftner, Dillner, & Dillner, 2007).

It is estimated that some 10% of sexually active women, with normal cervical cytology, are at risk of developing persistent infection (de Sanjose et al., 2007). These women represent a high-risk group for initiation of a pathological process with progression to precancerous and invasive cancer lesions. In addition, smoking, sexual habits, parity, oral contraceptives and genetic factors have all been associated with increased risk ((Moreno et al., 2002; Moreno et al., 1995; Munoz et al., 2002).





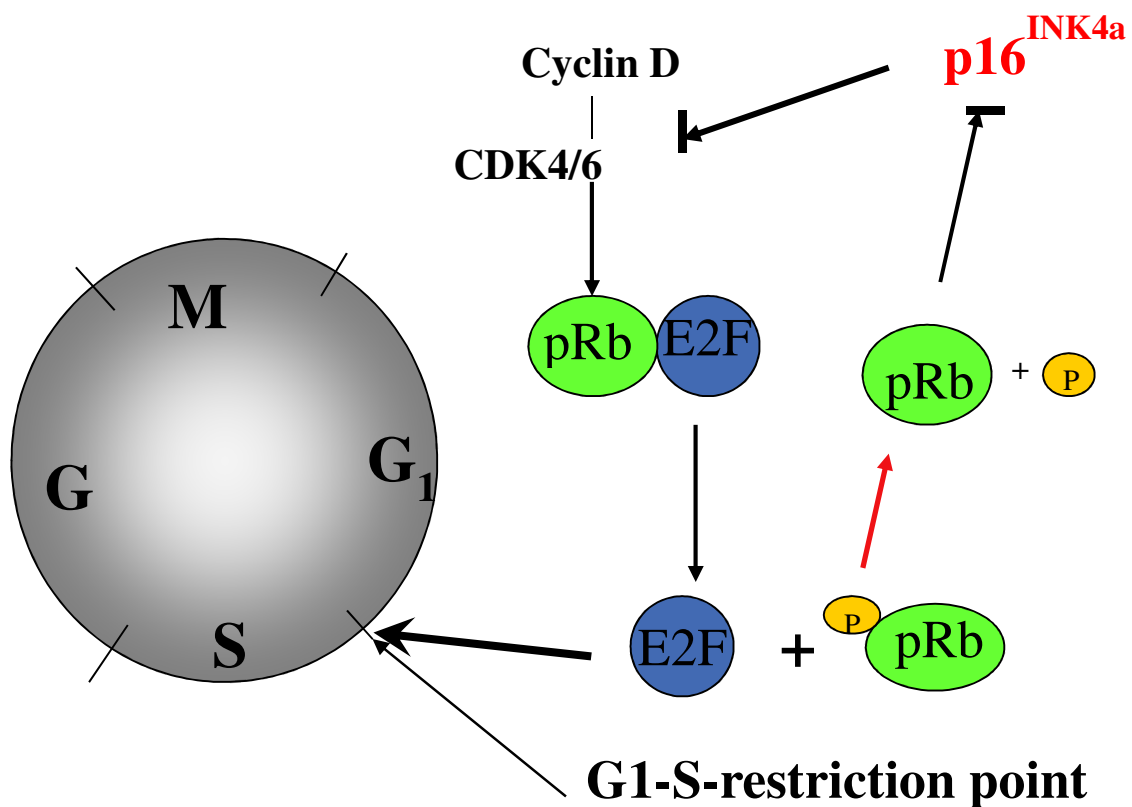
**Figure 8.** Squamous cells with high grade lesions.

#### **3.6.4. Onset of infection**

HPV infects basal cells, usually at the site of the transformation zone with its squamo-columnar junction; less commonly, the virus enters through a microscopic wound elsewhere in the epithelium. Studies have shown that the viral capsid protein L1 binds to syndecan-1, the heparan sulfate proteoglycan, which acts as the receptor to internalize the viral particle (Horvath, Boulet, Renoux, Delvenne, & Bogers, 2010; Johnson et al., 2009; Joyce et al., 1999). Other studies have shown that the minor capsid protein L2 also participates in this process of binding to the cell membrane (Bergant & Banks, 2013; Woodham et al., 2012). Production of virions depends on differentiated superficial epithelial cells, where L1 and L2 capsid proteins are produced.

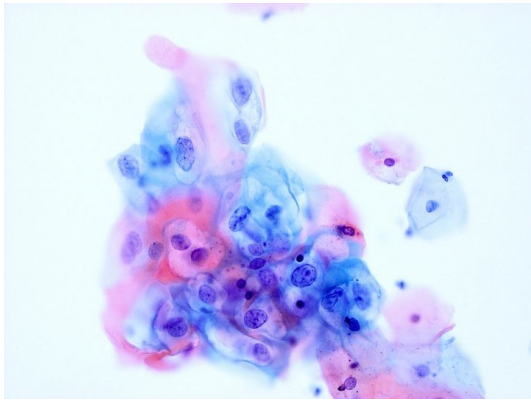
The virus infects basal cells as the primary target; after 12-24 hours (Maglennon, McIntosh, & Doorbar, 2011) replication produces about 200 viral DNA copies per cell. The infected cells enter a phase of episomal maintenance, during which time transcription from the two early genes, E6 and E7, is hardly measurable. Once the infected keratinocyte begins to differentiate, expression of E6 and E7 is high. When the cell exits the cell cycle, viral DNA replication continues, with amplification resulting in at least 1000 viral copies per cell. The primary cycle of infection is complete, after at

least 3 weeks, when the L1 and L2 capsid proteins are expressed in the upper epithelial layer, allowing the assembly and release of complete virions at the epithelial surface (M. Stanley, 2010). The function of E6 and E7 is to activate DNA synthesis during replication by interfering with the two tumor suppressor proteins, p53 and retinoblastoma protein (pRb) (Dyson et al., 1989). E6 protein increases degradation of p53, while E7 protein irreversibly phosphorylates pRb, thereby inactivating it and causing a release of transcription factor E2F. Since the two tumor suppressor proteins, p53 and pRb, function as “guardians of the genome,” long-term infection with HR-HPV may eventually affect cellular genetic stability by increasing the likelihood of survival for mutated cells. E6 and E7 also play a major role in carcinogenesis through evasion and downregulation of the innate immune response. The ability of the virus to evade the immune system is a prerequisite for tumorigenesis. (Tindle, 2002).



**Figure 9.** p16<sup>INK4a</sup> in normal cell. With permission from Dako Sweden.

### 3.6.5. Morphological changes due to HPV infection



**Figure 10.** Koilocytes. Mature squamous epithelial cell with a perinuclear cytoplasmic halo.

Indications of HPV infection may be ascertained from both cytological and histological samples. The most characteristic cytological finding of HPV is koilocytosis. The koilocyte is a mature squamous epithelial cell with a large perinuclear cytoplasmic halo. The halo may be irregular and surrounded by a dense cytoplasmic zone. (Frost, 1997; Papanicolaou, 1960). In 1976-77, two separate studies described the koilocyte as the most characteristic cytological feature of HPV infection (Meisels & Fortin, 1976; Meisels, Morin, Casas-Cordero, Roy, & Fortier, 1981; Meisels et al., 1981; Purola & Savia, 1977). Indeed, this finding represents a fixation artifact resulting from the effects of HPV on the cytoskeleton and shrinkage during dehydration; in cytology it is a strong indicator of HPV infection (Tanaka, Chua, Lindh, & Hjerpe, 1993).

### 3.6.6. HPV vaccines

Prophylactic HPV vaccines were developed to prevent HPV infections. Two such licensed HPV vaccines are currently available. The first, Gardasil® (Merck & Co, Whitehouse Station, NJ, USA), is a quadrivalent vaccine based on virus-like particles (VLP) from types 6, 11, 16 and 18, while the second, Cervarix® (GlaxoSmithKline Biologicals, Rixensart, Belgium) is bivalent, containing only VLPs from types 16 and 18. Both were approved for use in the US, Europe and Australia in 2006. Studies have shown almost complete protection against HPV infection from the types found in the

respective vaccines. The HPV vaccines are expected to provide more than 50% protection against ICC (B. Lu, Kumar, Castellsague, & Giuliano, 2011; "Quadrivalent vaccine against human papillomavirus to prevent high-grade cervical lesions," 2007). A study recently showed a decreased risk for condyloma after two of three doses of vaccine (Herweijer et al., 2014). Therapeutic effects on established CIN lesions have not been found.

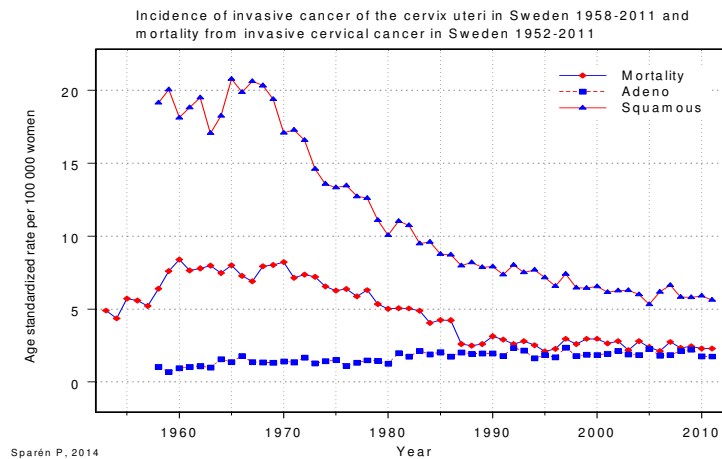
A nine-valent vaccine containing HPV types 6, 11, 16, 18, 31, 33, 45, 52 and 58 has been tested and is expected to provide 90% protection against ICC. (Serrano et al., 2014). However, this vaccine is not yet commercially available. The antibodies produced against HPV virus are present in the secretions covering the cervical canal, squamocolumnar junction and vagina, which is believed to contribute to the vaccine-mediated neutralization of the virus. In this way the vaccine may protect against infection in the cervix and vagina, but not on dry surfaces such as the vulva and penis. (M. A. Stanley, 2012).

### **3.7. CANCER PREVENTION**

#### **3.7.1 Cytology**

Carcinoma develops over an extended period of time, 10-20 years (H. C. Chen et al., 2011; McCredie et al., 2008), with premalignant stages that can be detected by cytology. (Echelman & Feldman, 2012; Russell, Raheja, & Jaiyesimi, 2013; Schmitt et al., 2013). Mortality in cxa has decreased in countries that have implemented a population-based cervical cytology screening program (Gustafsson & Adami, 1989; Gustafsson, Ponten, Bergstrom, & Adami, 1997). The main goal of population-based cervical screening is to reduce incidence and mortality from cervical cancer. The aim is detection and treatment of early lesions while they are still precancerous. This slow carcinogenic process meets the WHO criteria for mass screening using the Pap smear, Papanicolaou test, Pap test, or cervical smear, (G. N. Papanicolaou, 1942; G. N. Papanicolaou & Traut, 1997; Papanicolau GN, 1941) a comparatively simple and cost-effective approach. The Cytopathologist and innovator, father of cytology, Dr. George

Papanicolaou was first to introduce the Pap smear in the 1920s. It was through Dr Papanicolaou's effort that cytology became accepted as a diagnostic method.



**Figure 11.** Incidence och mortality of cxca in Sweden. With permission from Pär Sparén, Karolinska Institutet, Sweden.

Sweden first offered primary cytology screening programs for cervical cancer in Stockholm in 1967, and the program was recommended for nationwide implementation in 1973, using the conventional Pap smear (Socialstyrelsen.se, 1998). The program initially encompassed women aged 30 to 49, with a four-year sampling interval (Pettersson, Bjorkholm, & Naslund, 1985). At that time the oncogenic mechanisms were not yet well understood and the incidence of cxca exceeded 800 cases per year. The current recommendation in Sweden is to call women aged 23-49 for sampling every 3 years and women aged 50-60 every 5 years. (Socialstyrelsen.se, 1998).

A recent audit indicates that the screening program has been effective (Figure 11). Since its inception, the incidence of cervical squamous cell carcinoma (SCC) has decreased considerably (Bergstrom, Sparen, & Adami, 1999; Gunnell et al., 2007). It is important that the program reach the at-risk population, especially women older than 35 years. The majority of women diagnosed with advanced-stage invasive cxca have never participated in the screening program (Bos, Rebolj, Habbema, & van Ballegooijen,

2006; Rozemeijer et al., 2014). Reliability in cytological screening is dependent on repeated sampling; thus some cancers will develop despite previously normal screening results (Andersson-Ellstrom, Seidal, Grannas, & Hagmar, 2000; Andrae & Strander, 2000).

A prospective cohort study (n=1230) of all cervical cancers 1999-2001 showed that invasive cxcas detected by screening had a better prognosis than cancer detected by symptoms (Andrae et al., 2012). Despite the encouraging results of the screening program, cxcas are still responsible for many deaths (Hakama, 1993) (Andrae & Strander, 2000). Pap test sensitivity may be limited by inadequate sampling with low cellularity, poor cell preservation or suboptimal sampling with insufficient yield from the transformation zone. Sampling error is the main factor responsible for low sensitivity and contributes to a large proportion of false negative results (Chamberlain, 1986; Dunn & Schweitzer, 1981; Gay, 1984).

Annually, nearly 800,000 cytology samples are obtained in Sweden, with the largest laboratory reporting some type of change in 5-6% of these (Nationellt Kvalitetsregister för Cervixcancerprevention, 2014). This number is significantly larger than the number of women who would otherwise develop cxcas. While women with precancerous changes are successfully identified, these analyses also initiate a follow-up process for a number of women who are not likely to develop cxcas since minor changes may often be impossible to distinguish from true precancerous lesions.

Thus the moderate specificity associated with Pap smears (Andrae et al., 2008) may cause unnecessary anxiety for the latter group of women and significant costs for society. (Ostensson, Froberg, Hjerpe, Zethraeus, & Andersson, 2010; Ostensson et al., 2013).

### **3.7.2. HPV-screening**

The organized cxcas screening program has been successful, but several studies have shown that one single Pap test has a limited sensitivity, 50-70%, for detecting CIN (Andrae, et al., 2008; Dunn & Schweitzer, 1981). Today, the HPV testing is a

commonly used supplementary analysis to better separate reactive lesions from precancerous ones (Arbyn et al., 2012; Ronco et al., 2014). On the basis of the nearly 100% link between HR-HPV and cxa, testing for HPV DNA is now being established. The sensitivity for detecting a premalignant or established malignant lesion at least correspondingly to CIN2 (CIN2+) or CIN3 (CIN3+) lesion is very high. HR-HPV is present in about 95% of samples with CIN2+, but the specificity is low (Cuzick et al., 2006) (Arbyn et al., 2009; Franco, 2009). Around 60% of HPV positive women have no detectable lesion (Cuzick et al., 2003; Herbert, 2007).

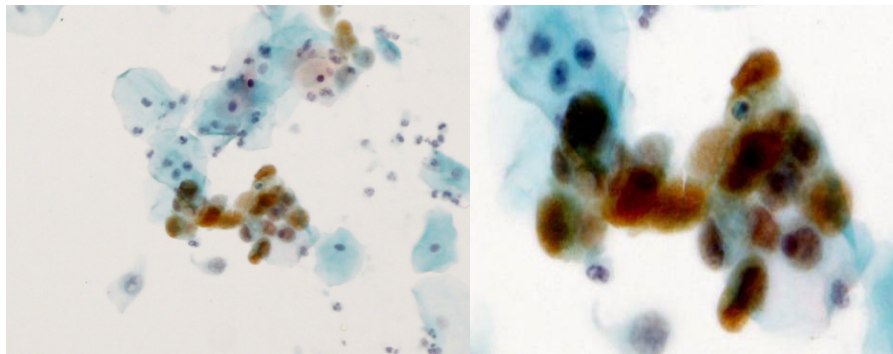
Today HPV testing is a commonly used supplementary analysis to help distinguish reactive lesions from precancerous lesions. In samples read as ASCUS, LSIL absence of HR-HPV indicates that the observed abnormality is unlikely to be precancerous (Kitchener, Denton, Soldan, & Crosbie, 2013; Naucler et al., 2007) and absence of HR-HPV indicates that an observed abnormality is less probably precancerous. Furthermore, HPV testing is sensitive as a “test of cure” after treatment of CIN2+ lesions, (Arbyn et al., 2004; Arbyn, et al., 2012; Chua & Hjerpe, 1996). LBC has an advantage in that adjuvant HPV testing can be performed as a “reflex test” from residual sample material, without having to recall the patient. New generation of HPV tests may be more specific and may distinguish persistent from transient HPV infection, although this is not yet the case. Together, LBC and HPV can increase the sensitivity and specificity of the test.

### **3.7.3. HPV testing in primary screening**

Studies have shown that, primary HPV screening can be effectively combined with cytology triage of HPV positive women (Kitchener, 2015; Kotaniemi-Talonen, Nieminen, Anttila, & Hakama, 2005). Moreover, some years later another study showed no different sensitivity in HPV and cytology screening (Kotaniemi-Talonen et al., 2007; Malila et al., 2013).

Lower costs for HPV testing today is another argument for primary screening (Bistoletti, Sennfalt, & Dillner, 2008; Ostensson, et al., 2010). Presence of unusual HR-HPV types not detected with today's commercial reagents will leave some cases undetected. Furthermore, studies have shown that HR-HPV screening can be as effective as cytology screening in reducing the incidence of precancerous abnormalities (Arbyn, et al., 2012; Ronco, et al., 2014). Several countries have or are in the process switching to primary HR-HPV testing, however, the primary screening for HPV remains controversial.

### 3.8. MOLECULAR MARKERS



**Figure 12.** p16<sup>ink4a</sup> reactivity in HSIL sample.

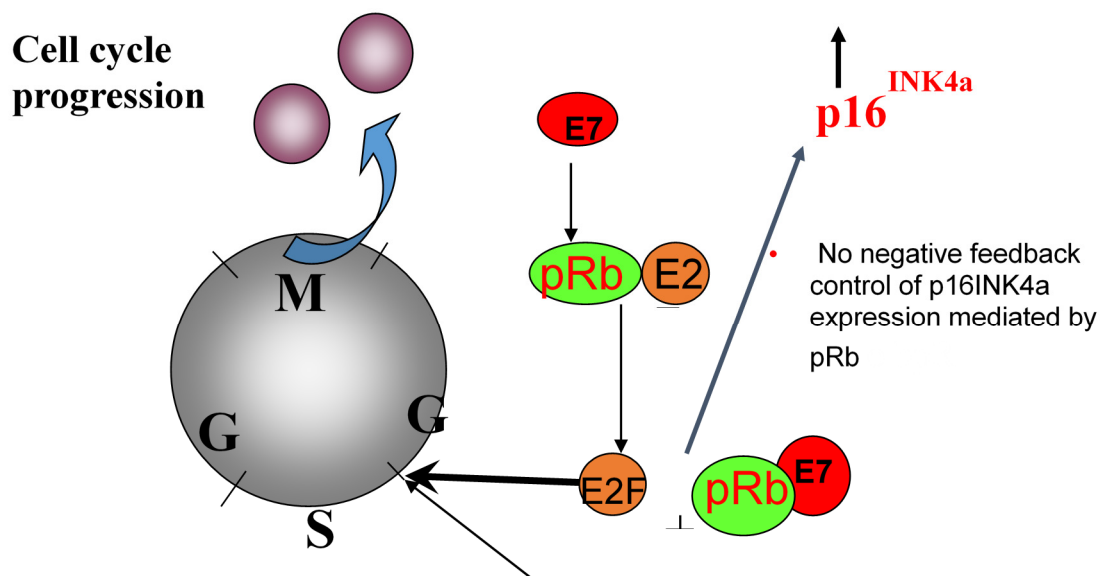
#### 3.8.1. p16<sup>INK4a</sup>

p16<sup>INK4a</sup> is a protein that acts as a cyclin-dependent kinase (CDK) inhibitor and cell-cycle regulator; expression is normally strictly controlled. Expression of p16<sup>INK4a</sup> prevents pRb phosphorylation, E2F release and cell cycle progression. Free E2F also stimulates p16<sup>INK4a</sup>, which in turn leads to reduced phosphorylation of pRb and therefore increased binding and inactivation of E2F. Active pRb is phosphorylated by the CDK complex and the subsequent release of E2F allows the cell to progress from G1 to S-phase. In normal cells (Figure 9), p16<sup>INK4a</sup> influences cell proliferation via a negative feedback loop to downregulate CDK 4 and CDK 6. This downregulation leads



to cell cycle arrest with accumulation of inactive unphosphorylated pRb-E2F complex and less free E2F transcription factor (Stern et al., 2012).

The effects of the E7 protein on pRb in the transformed cell are interaction and inactivation. E7 disrupts the inactive pRb-E2F protein complex, increasing free E2F, which in turn leads to binding of E7 to pRb protein (Figure 13). This degradation of pRb protein by E7 activates E2F transcription, resulting in increased free E2F which stimulates p16<sup>INK4a</sup> expression. Thus expression of E6 and E7 results in uncontrolled cell cycle progression without apoptosis, thereby increasing the risk for mutations. Therefore such expression is necessary for malignant transformation of the infected epithelium.



**Figure 13.** p16<sup>INK4a</sup> in oncogenic transformation. With permission from Dako Sweden.

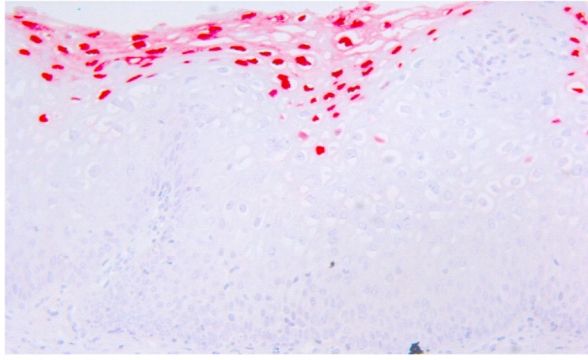
Accumulation of p16<sup>INK4a</sup> in the nucleus (Figure 12) is a consequence of impaired pRb and has therefore been proposed for use as a biomarker to identify dysplastic cells (von Knebel Doeberitz, 2002). In normal cells, p16<sup>INK4a</sup> expression is either faint or undetectable by immunocytochemistry. Some immunoreactivity to this protein can be seen during cellular stress, such as in reactive conditions and repair situations. In LBC samples p16<sup>INK4</sup> staining can be conducted for ancillary analysis, using leftover cell suspension. Immunocytochemical demonstration of p16<sup>INK4a</sup> using conventional Pap smear counterstaining technique may improve our ability to distinguish between premalignant and reactive cellular stress. (Paper III)

### **p16<sup>INK4a</sup> / Ki-67**

A combination of p16<sup>INK4a</sup> and Ki-67 is of interest for detection of exfoliated cells demonstrating simultaneous HPV transformation and proliferation. Immunocytochemical dual staining using the CINtec PLUS p16<sup>INK4a</sup> /Ki-67 kit (Roche) can be performed on LBC samples. Studies have shown that this staining technique improves detection of CIN2+ on ASCUS and LSIL samples (Roelens et al., 2012; E. M. Smith, Rubenstein, Hoffman, Haugen, & Turek, 2010). Another study based on cytology negative and HR-HPV positive women have shown that the use of this dual staining cytology, when used as a reflex test, may identify the vast majority (<90%) of underlying HSIL (Petry et al., 2011).

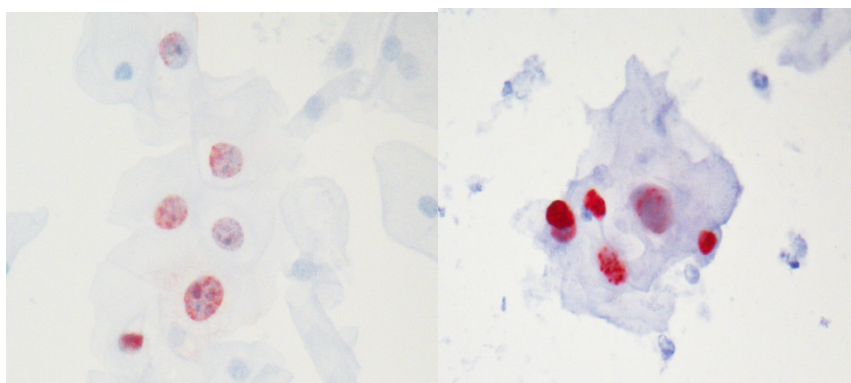
### **3.9.2. L1 capsid protein**

As previously mentioned, the major HPV L1 capsid protein is only produced during formation of HPV virions and only in the superficial squamous cell layer (Figure 13). The L1 and L2 capsid proteins are immunogenic and may, when released, initiate an immunological response.



**Figure 13.** HPV L1 reactivity only in the superficial squamous cell layer.

Expression of this protein may indicate the possibility of spontaneous regression of the dysplastic lesion and the L1 protein may therefore serve as a prognostic marker. (Griesser, Sander, Hilfrich, Moser, & Schenck, 2004; Hilfrich & Hariri, 2008; Melsheimer, Kaul, Dobeck, & Bastert, 2003; Rauber, Mehlhorn, Fasching, Beckmann, & Ackermann, 2008), (Mehlhorn et al., 2014). The immune response to the L1 capsid protein (Figure 14), on which the available vaccines are based, may initiate clearance of the infection and prevent it from being persistent.



**Figure 14.** Week to moderate L1 reactivity (left) and strong L1 reactivity (right).

Accordingly, absence of L1 expression may be one way by which infected cells are able to avoid the T cell-mediated immune response, which may result in persistent infection (M. Stanley, 2006). Studies have shown that immunocytochemical staining of the L1 capsid protein may be valuable for predicting early precancerous lesions (Griesser, et al., 2004), (Hilfrich & Hariri, 2008). Furthermore, in another study of women with LSIL and HR-HPV, absence of L1 reactivity predicted both increased likelihood of progression and decreased likelihood of regression, compared with women in whom this protein was expressed. L1 reactivity has also been shown to have similar predictive value in CIN1 and CIN2 lesions (Rauber, et al., 2008).

### **3.9.3. microRNA**

Recent advances in molecular biology and gene technology have opened up new opportunities to improve the yield of diagnostic information from sampled material. LBC samples are preferable when using these techniques. Many genetic alterations have been identified in cxc, but little is yet known about the role of microRNAs (miRNAs) in carcinogenesis. In most tumors, levels of many miRNAs are reduced, with the lowest levels found in the least differentiated tumors (J. Lu et al., 2005; Lui, Pourmand, Patterson, & Fire, 2007). MicroRNAs represent a family of short single-stranded noncoding RNAs, with about 22 nucleotides. Many functions have been attributed to them.

Experimental studies have shown that miRNAs are regulators of important biological processes such as cell proliferation, apoptosis, viral infection and cancer development (Bartel, 2004). Several studies have shown that miRNAs are aberrantly expressed or mutated in tumors and data strongly suggest that miRNA profiling is more robust than mRNA profiling with respect to tumor classification (Calin & Croce, 2006; Di Leva, Calin, & Croce, 2006; Garzon, Fabbri, Cimmino, Calin, & Croce, 2006). Studies have also shown that miRNA with altered expression has been identified in cxc when compared with normal cervical tissue (Huang et al., 2012; Lee et al., 2008; Lui, et al., 2007; Martinez et al., 2008; Wang et al., 2009; Witten, Tibshirani, Gu, Fire, & Lui,

2010). In addition, a recent study confirmed that miR-205 expression was frequently higher in SCC than in normal cervical tissue (Xie, Zhao, Caramuta, Larsson, & Lui, 2012).

## **2 4. AIMS**

### **4.1. GENERAL AIM**

The primary aim of this thesis is to explore what diagnostic information can be obtained from a single sample in the fields of cytology, virology and molecular biology using LBC. Would such a combination of analyses improve our ability to identify women at risk of developing cxca?

### **4.2. SPECIFIC AIMS**

#### **4.2.1. Paper I**

This split-sample study is designed to identify whether LBC provides higher sensitivity as a screening method for precancerous abnormalities compared with conventional Pap smear.

#### **4.2.2. Paper II**

The purpose of this study was to compare the combination of LBC plus HPV reflex testing in cases of minor cytological abnormalities (LBC+HPV testing) with conventional cytology (CC) in regard to their ability to identify precancerous lesions in a population-based screening setting.

#### **4.2.3. Paper III**

The aim of this study was to evaluate the use of immunocytochemical demonstration of p16<sup>INK4a</sup> as a diagnostic marker in LBC samples and to assess the potential for this marker to distinguish between reactive and precancerous conditions.

#### **4.2.4. Paper IV**

The aim of this study was to evaluate the prognostic relevance of HPV L1 capsid protein expression and the presence of different HR-HPVs in LBC samples from women with minor cytological abnormalities.

#### **4.2.5. Paper V**

The purpose of this study was to determine whether the level of miR-205 expression in LBC samples correlates with the presence of HR-HPV, histology and predicts progression.

## **3 5. MATERIAL AND METHODS**

### **5.1. ETHICAL PERMISSION**

All studies were approved by the regional Ethics Review Board in Stockholm and informed consent was obtained from all study subjects.

Paper I. 04-679/3, Paper II. 04-679/3, Paper III. Dnr.04-679/3, 2010/944-32,

Paper IV. Dnr.04-679/3, 2010/944-32,

Paper V. Dnr.04-679/3, 2010/944-32, 2013/542-32

### **5.2. STUDY DESIGN**

Paper I, II, III, IV, V

LBC samples were obtained from the primary screening program, either as the primary sample or during secondary follow-up of abnormal findings within the program. The women were examined 2-6 months later at the Department of Gynecology and Obstetrics at Karolinska University Hospital Huddinge, Stockholm with pelvic examinations including cytology and histology sampling, colposcopy and biopsy. The cytology samples were independently evaluated, in accordance with the Swedish classification system, by different cytotechnologists and cytopathologists. The histological samples were assessed by a local pathologist and classified according to the WHO CIN classification, to be used as the gold standard.

#### **5.2.1. Paper I**

The study enrolled 137 women with any grade of cytological abnormality detected through the population-based cervical screening program in 2004. The split-sample study design allowed the performance of the two cytological methods to be evaluated through use of paired specimens. After first obtaining a conventional Pap smear, the remaining material was then rinsed in the LBC vial (ThinPrep®).

#### **5.2.2. Paper II**

After evaluating the diagnostic performance from the first study, Paper I, a total of 8320 women from the population-based primary cervical screening program were invited to



participate in the study in 2005 and 2006. Six sampling centers in southern Stockholm participated in the study and the midwives involved had no influence on sample interpretation. Depending on the week of the appointment, either conventional Pap smear or LBC (ThinPrep®) samples were obtained and sampling technique was changed each week. In all, 4261 women were assigned to Pap smear and 4059 to LBC sampling. All women with any grade of abnormality were referred for colposcopy with pelvic examination, cytology resampling and histology sampling. In cases where low-grade cytological abnormalities (ASCUS and LSIL) were found, supplementary HPV DNA testing was performed using LBC. Follow-up was undertaken on all women with abnormal cytology and test results were compared with histology and HPV testing within two years after the first cytology sampling.

### **5.2.3. Paper III**

We consecutively enrolled 118 women with any grade of cytological abnormality detected through the population-based primary cervical screening program in 2004. In 2005 these women were called for follow-up testing and immunocytochemical staining was performed with LBC samples and compared with the histological findings that served as the gold standard.

### **5.2.4. Paper IV**

This study enrolled 112 women with minor cytological abnormalities detected through the population-based primary cervical screening program in southern Stockholm, Sweden between 2007 and 2009. All LBC samples were collected by midwives and prepared and evaluated at the Department of Clinical Pathology and Cytology at Karolinska University Hospital, Stockholm. Immunocytochemical staining for the HPV L1 capsid protein was performed according to manufacturer protocol and the results compared with the cytological and histological findings.

### **5.2.5. Paper V**

We enrolled 127 LBC samples classified as WNL, LSIL, HSIL, ICC that were obtained through a population-based primary cervical screening program in Stockholm and from the Department of Gynecology and Obstetrics at Karolinska University Hospital, Stockholm, between 2008 and 2011. The present study evaluated levels of miR-205 expression and compared the findings with HPV type and histological lesion.

### **5.3. PREPARATION OF CYTOLOGY SAMPLES**

*Conventional Pap smear, (Papers I, II):* The cells were collected from the fornix, ectocervix and endocervix using conventional Pap smear technique with a spatula and cervical brush. The cell sample was smeared into a glass slide and was immediately fixed in 95% ethanol, air-dried and stained according to protocol (G. N. Papanicolaou, 1942) and interpreted by cytotechnologists.

*LBC, Papers I, II, III, IV, V:* First a conventional Pap smear was obtained and the remaining material from spatula and brush was rinsed off into a vial with PreservCyt solution (ThinPrep®, Hologic, Marlborough, Ma USA). All LBC samples were prepared using a ThinPrep® 2000 processor (Hologic, Marlborough, Ma USA). All glass slides were stained according to a modified Pap protocol (more concentrated acetic acid) and evaluated by different cytotechnologists. Different individuals independently assessed the samples according to the Bethesda classification, modified to Swedish recommendations.

### **5.4. HISTOLOGY**

In all Papers (I, II, III, IV, V) histological biopsies and cervical cones were performed using Zeiss OMPI colposcopy (Zeiss, Oberkochen, Germany) for magnification. The samples were preserved in 4% formaldehyde, analyzed according to the laboratory protocol and diagnosed and assessed by pathologists according to the CIN classification system ((Fu, et al., 1981). Histological results were retrieved from medical and laboratory records and from the Regional Cancer Center in Stockholm.

### **5.5. HPV GENOTYPING. (PAPERS. II, III, IV, V)**

### **Linear Array HPV Genotyping: Papers II, III, IV.**

HPV DNA genotyping was performed on 2 ml of residual LBC cell suspension taken from LBC samples that showed ASCUS or LSIL. The cell suspension was centrifuged and lysed using the Total Nucleic Acid Isolation kit (Roche, Basel, Switzerland). DNA was extracted using the MagNA Pure LC Robot (Roche) and analyzed with the Linear Array HPV Genotyping Test (LA) (Roche) according to manufacture protocol.

LA is a qualitative genotyping assay for 37 HPV types based on polymerase chain reaction (PCR) and probe hybridization. There are 12 HR-HPV types: HPV16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, and 59; six probable HR-HPV (pHR-HPV) types: HPV26, 53, 66, 68, 73, and 82; and 19 LR-HPV types: HPV6, 11, 40, 42, 54, 55, 61, 62, 64, 67, 69, 70, 71, 72, 81, 83, 84, IS39, and CP6108. The LA test uses biotinylated primers during amplification of a 450 bp region on the HPV L1 gene, using a 268 bp region on human beta globin as a control.

### **PapilloCheck® HPV Test. Paper V**

**The PapilloCheck® HPV Screening test** (greiner-bio-one), which is based on detection and genotyping of a sequence of the viral E1 gene, can identify 24 types of papillomavirus. The analysis is capable of showing the presence of 18 HR-HPV types (16, 18, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, 70, 73, 82) and six LR-HPV types (6, 11, 40, 42, 43, 44). The DNA was extracted using the PapilloCheck® HPV Extraction Kit, and a 350 bp fragment of the E1 gene was obtained with multiplex-PCR. The human “housekeeping gene” ADAT1 (Adenosine deaminase tRNA-specific1) was used to show the presence of intact DNA. The amplification products were hybridized to specific probes attached to the chip. During hybridization, the bound DNA was fluorescently labelled and unbound DNA removed by washing. Finally, the PapilloCheck® DNA-chip was automatically scanned, analyzed and evaluated using the CheckScanner™ and CheckReport™ Software respectively.

## **5.6. IMMUNOCYTOCHEMISTRY**

In **Papers II and IV**, extra slides from the remaining LBC sample were prepared for immunocytochemical staining according to manufacturer protocol.

### **5.6.1. p16<sup>INK4a</sup> (Paper III)**

The slides were post-fixed in acetone (PA) for 10 minutes at room temperature and air-dried at least 30 minutes prior to immunostaining. All slides were subjected to antigen “Heat Induced Epitope Retrieval” (HIER) using Epitope Retrieval Solution (Dako, Copenhagen, Denmark) at 95°-100°C for 10 minutes with Tris/EDTA buffer pH9. Immediately thereafter, the Coplin was removed from the water bath and left at room temperature for at least 20 minutes, followed by washing in diluted wash buffer (DakoCytomation) for 5 minutes. p16<sup>INK4a</sup> staining was performed with the DakoCytomation Autostainer using the CINtec™ p16<sup>INK4a</sup> Cytology Kit K5339, (Dako Cytomation, Glostrup, Denmark), according to the DakoCytomation protocol.

For each staining, at least one positive and one negative control was used. Counterstaining was performed according to the Papanicolaou protocol using the LEIKA ST4040 automatic staining machine. Staining does not interfere with the immunocytochemistry, making it possible to simultaneously assess both dysplasia and p16<sup>INK4a</sup> reactivity. The slides were analyzed by light microscopy and independently assessed by 2 people. p16<sup>INK4a</sup> nuclear reactivity was considered to be negative (-) if fewer than three cells per slide showed reactivity (Bibbo, Klump, DeCecco, & Kovatich, 2002). Intensity of nuclear staining was evaluated according to increasing p16<sup>INK4a</sup> reactivity (+, ++, +++).

### **5.6.2. HPV L1 capsid protein (Paper IV)**

HPV L1 staining was carried out using the specific monoclonal antibody, Cytoactive®, (Cytoimmun, Diagnostics GmbH, Pirmasens, Germany) according to manufacturer’s protocol. The slides were fixed in 96% ethanol for 20 minutes, air-dried and stored at

room temperature until immunostaining was carried out. Antigen retrieval was performed using microwave treatment in Citrate buffer pH6 for 20 minutes. The Cytoactive® screening antibody was directly added to the slides and incubated at room temperature. The slides were then counterstained with HTX and L1 reactivity was independently assessed by different people. Slides in which at least one cell showed nuclear L1 reactivity (Griesser, et al., 2004) were scored as positive.

## **5.7. QUANTITATIVE REVERSE TRANSCRIPTION PCR (QRT-PCR)**

*MicroRNA extraction.* The microRNA study was carried out using 2 ml of residual cell suspension from each LBC sample. miRNA extraction was carried out using the mirVana™ miRNA isolation kit (Applied Biosystem/Ambion, Austin, TX, USA), applying small RNA enrichment from the LBC samples. Isolated miRNA concentration was measured with a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE).

*Reverse transcription:* For mature miRNA, total RNA was reverse-transcribed to cDNA using the TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems). The Reverse Transcription Reaction Master Mix was prepared for the target miRNA and the endogenous control RNU6B.

*qRT-PCR:* qRT-PCR reactions were performed in triplicate and expression of mature miRNA was quantified using the Applied Biosystems 7500 Fast Real-time PCR system (Applied Biosystems).

## **5.8. STATISTICAL ANALYSES**

Data were analyzed with Statistica software (Statsoft® Inc, Tulsa, OK, USA). SAS® System 9.1 (SAS Institute Inc., Cary, NC, USA) software was used.

### **5.8.1. Paper I**

The Chi-square ( $\chi^2$ ) statistic was calculated to test the significance of the data. All statistical tests were two-sided and the null hypothesis of difference was rejected at a significance level of  $p < 0.05$

### **5.8.2. Paper II**

Logistic regression was used to assess the rates of CIN detection and of abnormal cytology in both the LBC-HPV triage group and the conventional cytology group. Results were presented as odds ratios (OR) and 95% confidence intervals (CI). A multivariate logistic regression model was computed including potential confounders. The times when analyses were undertaken were included to account for possible differences related to the learning curve associated with LBC.

### **5.8.3. Paper III**

The chi-square test and Spearman rank-order correlation test were used to analyze results from immunocytochemical staining and CIN grading. Cytological and histological outcomes were computed to include sensitivity, specificity, positive and negative predictive value.

### **5.8.4. Paper IV**

Data were analyzed using frequency count and percentages. The chi-square test was used to analyze the association between L1 capsid protein expression, different HR-HPV types and CIN grade.

#### **5.8.5. Paper V**

The association between miR-205 expression and HPV status was analyzed with the Mann-Whitney U-test with continuity correlation. The association between miR-205 expression and diagnosis (including cytology and histology) was analyzed by one-way ANOVA Kruskal-Wallis test. The correlation between miR-205 expression and age was analyzed using the Spearman rank-order correlation test. All statistical tests were two-sided and the null hypothesis of difference was rejected at a significance level of  $p < 0.05$ .

## 4 6. RESULTS

### 6.1. PAPER I

#### **A comparison of liquid-based cytology and Pap smear as a screening method for cervical cancer.**

Results from screening 137 Pap smears showed: ASCUS in 11 (8%) samples, LSIL in 40 (29%), HSIL, ASC-H, and SCC in 56 (41%), while two samples showed atypical glandular lesions (AGUS). Twenty-eight (20%) samples were considered to be WNL. Samples prepared using LBC technique showed fewer cases of ASCUS, (6 cases, 4%), while LSIL was found in 44 (32%) samples, and HSIL and more advanced lesions in 60 (44%). AGUS was found in 2 samples, while 25 (18%) LBC samples were considered to be WNL. CIN2+ cases were more common, while ASCUS was less common compared with Pap smear results (**Figure I, Paper 1**)

The 28 (20%) women with normal Pap smears underwent simultaneous biopsy, with results showing CIN2+ lesions in 12 (43%) cases. Correspondingly, of the 25 benign LBC samples, 9 (36%) had CIN2+ lesions on biopsy. Of the 109 Pap smears with abnormal findings of any grade, 65 (60%) cases showed CIN2+ lesions, while of 112 LBC samples, 68 (61%) had CIN2+ lesions (**Table I and II, Paper 1**). The sensitivity for detecting CIN2+ lesions was 47% for Pap smear and 66% for LBC. Concordance with histological findings was 37% for Pap smear, while the corresponding figure for LBC was 53%; the difference was statistically significant ( $p=0.011$ ).

### 6.2. PAPER II.

#### **Liquid-based Cytology with HPV Triage of Low-grade Cytological Abnormalities Versus Conventional Cytology in Cervical Cancer Screening.**

In this study, histological diagnoses were available for about 80% of all women, with reported cytological abnormalities. Similar detection rates for pathological findings between LBC+HPV triage and conventional cytology (Pap smear) in cases where



CIN2+ and CIN3+ were confirmed during introduction of the LBC technique. When LSIL was diagnosed among HR-HPV negative women the rate of abnormal cytological findings was significantly higher using LBC+HPV triage compared with conventional cytology (Pap smear). However, when LSIL was not diagnosed among HR-HPV negative women (whose findings were diagnosed as WNL), total abnormal findings were lower using LBC+HPV triage compared with conventional cytology (Pap smear). The study reflects the learning process that occurs when implementing new laboratory technology. Our study may have an impact on performance thus staff at all levels (midwives, cytotechnologists and cytopathologists) had extensive experience with the conventional screening, but limited experience with the slightly different criteria of LBC. The performance of LBC improved over time and similar with the Pap smear. The improved attention in the cytology interpretation may have had an impact of both screening performances.

### **6.3. PAPER III**

#### **p16INK4a immunocytochemistry in liquid-based cervical cytology: Is it feasible for clinical use?**

Of 118 LBC samples, 111 (94%) had sufficient material for immunocytochemistry, from which five were excluded due to insufficient histological follow-up, leaving a total of 106 samples for immunocytochemistry. Of these 106 LBC samples, 13 (12%) were classified as WNL, 40 (37%) showed LSIL and 49 (46%) showed HSIL. Two LBC samples had ASCUS and one was classified as AIS.

Of 106 LBC samples, 10 (9%) showed strong p16<sup>INK4a</sup> reactivity, 28 (26%) moderate and 19 (18%) showed weak p16<sup>INK4a</sup> reactivity, while 68 (64%) of the total of 106 cases showed weak or no reactivity (**Tabel II, Paper 3**).

The intensity of p16<sup>INK4a</sup> reactivity correlated with the cytology classification. In one normal case and in all cases with low-grade lesions, there was weak reactivity to p16<sup>INK4a</sup>. Thirty-four of 49 (69%) HSIL samples showed moderate or strong reactivity.

Sensitivity for detecting HSIL with p16<sup>INK4a</sup> staining was 0.60 (CI: 95%; 0.50-0.70) and specificity was 1.0 (CI: 95%; 0.77-1.0); PPV was 1.0 (CI: 95%; 0.92-1.0)

Histological follow-up of the 106 cervical lesions showed benign findings in 19 (18%) cases, CIN1 in 26 (25%), CIN 2 in 28 (26%), CIN3 in 30 (28%) SCC in 1 case, while two were classified as AIS. The degree of p16<sup>INK4a</sup>.reactivity was also correlated to histological CIN classification (**Table I, Paper 3**). Sensitivity for detecting CIN2+ with p16<sup>INK4a</sup>.immunocytochemical staining was 0.59 (CI: 95%; 0.49-0.69), specificity was 0.94 (CI: 95%; 0.68-1.0), and PPV was 0.98 (CI: 95%; 0.89-1.0).

#### **6.4. PAPER IV**

##### **High-risk HPV L1 capsid protein as a marker of cervical intraepithelial neoplasia in high-risk HPV-positive women with minor cytological abnormalities.**

Of 112 LBC samples from women with minor cytological abnormalities, 108 (96%) had sufficient residual cell suspension for immunocytochemistry. Four samples were excluded due to insufficient material.

*Histology:* 23 (22%) cases were classified as WNL, 43 (41%) as CIN1, 23 (22%) as CIN2, and 15 as CIN3+ (**Table III, Paper 4**).

The most common HPV type was HPV16, found in 33% of samples, followed by HPV51 (20%), HPV31 (16%), HPV52 (11%) and HPV56 (11%). Multiple infections were found in 28 women.

Among HR-HPV-positive women, 48 (46%) were positive for L1 protein, while the remaining 56 samples (54%) were L1-negative. Expression of L1 protein varied among different HPV types. The HPV types that expressed L1 most frequently were HPV56 (80.0%), HPV45 (60.0%), HPV52 (58.3%) and HPV51 (52.4%) (Table II, In the Paper 4).

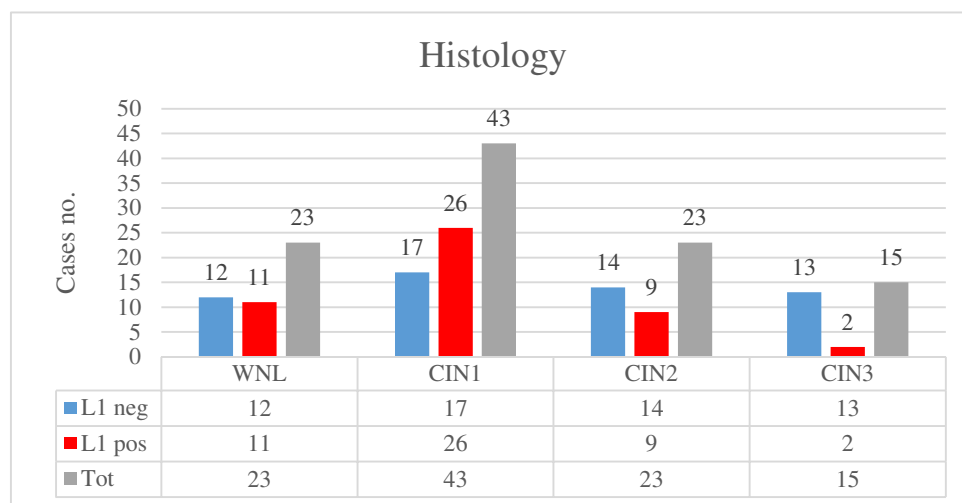
*L1 expression by histological result:* Reactivity to HPV L1 capsid protein was found in 13 of 23 (56%) WNL samples, 26 of 43 (60%) CIN1 samples and 11 of 38 (29%) CIN2+samples. Thus expression of L1 correlates negatively with CIN grade ( $p=0.012$ ) by Pearson's Chi-square test (**Table II**). In a univariate logistic regression model using

CIN2+ as an outcome, absence of HR-HPV L1 expression at enrolment was a predictor, with an OR of 3.2 (95% CI, 1.081-9.417) for progression to CIN2+.

Progression to CIN2+ among L1-positive women infected with HR-HPV, including HPV16, was found to be 35.3%, compared to only 15.4% in the group infected with both HR- and LR-HPV types (Table IV in Paper 4) stepwise logistic regression analysis shows (OR 4.46, CI, 1.4-14.212; P=0.0115). Among 13 HPV16-positive CIN1 cases, L1 expression was found in 8 (61.5%), whereas L1 expression was only in found in only 2 of 15 (13%) HPV16-positive CIN2+ cases (P=0.026).

We found that among women with CIN2+, HPV16 L1 expression was rare (13.3%). We show that 25.0% of women infected with HR-HPV only progressed to CIN2+. In the group of CIN2+ cases infected with both HR- and LR-HPV types, we found a much higher proportion of women that progressed to CIN2+ (35.7%) (Table V, Paper 4). In our limited material we showed that different HR-HPV types in combination with loss of L1 expression have different oncogenic potential for the development of CIN2+.

Tabel.II. HPV L1 reactivity correlated to histological diagnosis.



## 6.5. PAPER V

### Expression of microRNA in liquid based cytology, a possible prognostic marker for cervical cancer?

A total of 127 LBC samples were collected from women who participated in the cervical screening program between 2008 and 2012 in Stockholm. Among these samples, histology was available for 109 of 127 (86%) samples.

*Cytology:* Of 127 women, 19 (15%) cases were classified as WNL, 24 (19%) as LSIL, 77 (61%) as HSIL and 7 (6%) women had squamous cell carcinoma.

*Histology:* Histology showed 10 (9%) lesions classified as WNL, 21 (19%) as CIN1, 26 (24%) as CIN2, 42 (39%) as CIN3 and 10 (9%) women had squamous cell carcinoma.

The detailed information for each patient is listed in (**Table S1, Paper 5**). The summary of the clinical features is shown in (**Table 1, Paper 5**).

Frequency of HR-HPV DNA detection: One hundred samples out of 127 (78.7%) have available HPV screening information. In the study population 74 women were HPV-positive, of whom 68 (92%) had various HR-HPV types, while 42 (57%) had detectable HPV16 or HPV18. (**Table S1, Paper 5**).

Using relative quantification method ( $2^{-\Delta CT}$ ), as normalized to *RNU6B*, we calculate the relative expression of *miR-205* in the 127 liquid-based cytology samples. We performed Spearman Rank Order correlation analysis to investigate the associations of *miR-205* expression and ages. The study showed no significant correlation with age ( $R=-0.099$ ,  $P=0.27$ ) indicating *miR-205* expression were not associated with ages. By performing Mann-Whitney U-test, we analyzed the associations between *miR-205* expression and HPV presence in 100 available samples. There was no statistically significant difference between HPV-positive and HPV-negative samples ( $P=0.51$ ), indicating *miR-205* expression was not associated with HPV infection in cervical cancers. By performing one-way ANOVA Kruskal-Wallis test, we compared the expression of *miR-205* within different progression groups based on cytology diagnosis, histology diagnosis and final diagnosis ( $P=0.98$ ,  $0.97$  and  $0.75$ , respectively). Unfortunately, we could not observe

significant difference between groups, indicating that *miR-205* expression alone could not distinguish the progression of cervical cancer in liquid-based cytology samples.

The study also evaluated the correlation between *miR-205* expression and various disease-related features (age, HPV, cytology, histology) by using the two-tailed chi-square test; no statistical differences were observed.

## 5 7. DISCUSSION

Organized cervical cancer screening based on Pap smears has considerably reduced the incidence of cancer, but sensitivity for detecting CIN is limited when using a single test. (Nanda et al., 2000). LBC technology has now become an established method for improving screening performance.

In study I sensitivity for detecting CIN2+ was greater in LBC samples (66%) than in conventional Pap smear (47%). Even though the LBC specimens were prepared from residual Pap smear material, LBC was more sensitive for the detection of HSIL, while reducing the rate of ASCUS findings. This improved diagnostic performance may relate to better preservation of cell morphology, as demonstrated in other studies (Hoelund, 2003; Schledermann, et al., 2006; Strander, et al., 2007). LBC methodology entails immediate fixation, which eliminates problems from drying artifacts, while filtration reduces obscuring blood and inflammatory cells, thereby improving performance. Correlation between histological diagnosis and LBC was 53%, but significantly lower for Pap smear, only 37%.

Encouraged by these results, we carried out prospective performance testing using a test design similar to that used by others (Strander, et al., 2007). Thus samples from the population-based cervical screening program were randomly obtained either through conventional Pap smears or through LBC samples, culminating in two groups, each with over 4000 samples. When ASCUS or LSIL were identified in the LBC group, supplementary HPV DNA genotyping was obtained. Unlike previous studies (Kotaniemi-Talonen, et al., 2005; Strander, et al., 2007), our study showed no significant difference in screening performance between the two groups (, with similar detection rates and PPVs for high-grade lesions.

This study, however, was conducted shortly after the introduction of LBC technique; consequently the results are likely to reflect the learning curve. Initially, when the study began and despite years of experience of conventional screening, our cytotechnologists and cytopathologists had limited experience with this new technique.

At first, subtle LBC findings were difficult to recognize. Nevertheless, this new technique was introduced with no negative impact on laboratory performance. Interestingly, improved performance was seen with both LBC and Pap samples over time. One reason may be the interest generated in the new technology, resulting in discussion among staff regarding the enhanced nuclear and cytoplasmic morphology. LBC preparations, with more even distribution and fewer overlapping cells are easier to interpret. We also found that continuous monitoring of diagnostic performance is important in order to balance the risk of over-diagnosis with over-confidence at the other end of the spectrum. Over-diagnosis, associated with reactive or degenerative conditions, is a minor issue given the availability of supplementary HPV DNA testing. Effective use of complementary HPV analysis can improve specificity for identifying women at risk of developing cxa.

Modern technology allows us to tolerate a higher level of low-grade cytological screening abnormalities because LBC and HPV triage improve sensitivity for detection of CIN2+ (Arbyn, et al., 2004). The negative predictive value of HR-HPV analysis is very high; the virtual exclusion of any risk of precancerous lesions in HPV-negative cases is unlikely to decrease sensitivity for CIN2+ detection (Froberg, Johansson, Hjerpe, & Andersson, 2008). Although implementation of LBC did not initially improve screening performance, it allows for ancillary analyses that make it unnecessary to call women back for additional sampling, thereby avoiding added costs as well as many of the errors associated with repeat sampling.

The subsequent studies aimed to evaluate potential diagnostic and prognostic markers. The “reflex test” concept makes it possible to demonstrate the presence and elevated expression of the p16<sup>INK4a</sup> protein. Accumulation of p16<sup>INK4a</sup> in the cell nucleus results from impaired retinoblastoma protein (pRb) function. The E7 protein from HR-HPV disrupts pRb function, resulting in free E2F that stimulates expression of p16<sup>INK4a</sup>. Therefore the resulting accumulation of p16<sup>INK4a</sup> protein has been proposed as a biomarker to identify dysplastic cells that may indicate a premalignant condition. Thus overexpression of p16<sup>INK4a</sup> protein in dysplastic cells indicates active expression of HR-

HPV oncogene E7. This protein could therefore serve as a surrogate marker for oncogene expression in precancerous lesions.

Accumulation of p16<sup>INK4a</sup> can be demonstrated by immunocytochemistry and may improve the potential to distinguish between premalignant and reactive cells (Bibbo, et al., 2002; Klaes et al., 2001). We wanted to evaluate the potential to make this distinction using immunocytochemistry with Papanicolaou as counterstain. While most dysplastic cells showed strong p16<sup>INK4a</sup> antibody reactivity, reactivity in metaplastic and glandular cells was weak (Negri et al., 2006). Such weak reactivity is more likely to be associated with reactive cellular stress than with a premalignant process.

The study also showed that the intensity of p16<sup>INK4a</sup> reactivity correlated with CIN classification as assessed by both histology and cytology. Strong reactivity was associated with high-grade lesions, while only one of thirteen cases assessed to be WNL showed any reactivity, and only on a weak level, thereby indicating a high PPV for this technique. Conversely, absence of p16<sup>INK4a</sup> reactivity was seen in only 5 of 49 HSIL cases, indicating high sensitivity for detection of precancerous lesions. Absence of reactivity may indicate absence of cells expressing E7 in the sample.

Two different p16<sup>INK4a</sup> kits from the same manufacturer were assessed. After testing the first immunocytochemical staining kit, we tested the second one, purported to be more sensitive. We tested 37 samples with mild to moderate cytological abnormalities. Although the second kit showed slightly stronger immunoreactivity in some cases, despite the improved reagents the detection rates remained unchanged.

Demonstration of p16<sup>INK4a</sup> protein may prove useful as an ancillary screening test to indicate HPV DNA activity in premalignant changes. Sensitivity, however, may be too low to replace HPV DNA testing. A possible alternative role for this test may be to identify HR-HPV infections that are not yet oncogenically active. Such an application has been suggested for ASCUS- or LSIL-positive samples in combination with positive HPV triage, to help avoid unnecessary follow-up of negative cases (Carozzi et al., 2008;



Carozzi et al., 2013; Cuschieri & Wentzensen, 2008; Wentzensen & von Knebel Doeberitz, 2007).

A more recent development is to combine p16<sup>INK4a</sup> and Ki-67 in a dual-staining process. Currently, this immunocytochemical combination has been more thoroughly investigated than the initial studies based on p16<sup>INK4a</sup> single-staining (Bergeron et al., 2010; Denton et al., 2010; Trunk et al., 2004). Single-staining requires morphologic interpretation of immunoreactive cells to distinguish cells showing dysplasia from other reactive processes such as samples showing squamous metaplasia and endocervical cells (Roelens, et al., 2012). The p16<sup>INK4a</sup> /Ki-67 dual-staining technique has also been suggested as a novel approach to screen for precancerous lesions in young HR-HPV positive women (Denton, et al., 2010; Ikenberg et al., 2013; Petry, et al., 2011; Wentzensen et al., 2014).

In addition, the HPV L1 capsid protein has previously been shown to be a potential prognostic marker. This protein is produced in the superficial squamous cell layer during productive HPV infection (H. zur Hausen, 2002). Expression of this protein may signal possible spontaneous regression of the dysplastic lesion (Mehlhorn, et al., 2014). The L1 protein may initiate clearance of the infection and prevent it from becoming persistent. Absence of this protein expression may indicate successful avoidance of the innate immune response by infected cells, thereby enabling persistent infection which contributes to carcinogenesis. Earlier studies have shown (Grapsa et al., 2014; Griesser, et al., 2004; Hilfrich & Hariri, 2008) that immunocytochemical assessment of HPV L1 protein status may be important to predict outcome of minor cytological abnormalities and may perhaps serve as a better indicator of risk than presence of HR-HPV DNA.

For ethical reasons, it is not possible to monitor the importance of L1 expression over time by registering outcomes of infection. When evaluating the importance of HPV L1 expression in minor cytological abnormalities, we found that protein expression correlates negatively with CIN grade, which is even more apparent during infection with the most oncogenic HPV types. L1 expression was commonly found in normal and LSIL samples, but rarely in histologically confirmed high-grade lesions. Of 13 L1-

positive women infected with HPV16, only two (15%) progressed to CIN2+, while the corresponding rate among L1-negative women with HR-HPV was 35%. Among samples containing HPV16, (**Paper IV**), L1 reactivity was found in 62% of cases where the reading was CIN1, while the corresponding frequency was significantly less, 13%. A trend was also found indicating higher expression of L1 protein in cases of simultaneous infection with HR-HPV and LR-HPV.

The risk of developing CIN2+ was similar among L1-positive women co-infected with both HR-HPV and LR-HPV types and among those infected with HPV16 alone. Another study showed similar findings (Luostarinen et al., 1999). The low proportion of CIN2+ (15%) among L1-positive cases infected with HPV16 is noteworthy considering that HPV16 is one of the most oncogenic types. While 71% of L1-negative cases progressed to CIN2+, only 29% of all L1-positive cases did so. Interestingly, since L1 expression indicates productive infection, it may be postulated that the cells harbor the HPV virus episomally, without integration into the human genome. Once virions are released they may be exposed to the immune system, hypothetically producing an immune response and clearance of infection. In such a scenario, L1 capsid protein expression would have prognostic value.

Simultaneous immunocytochemical demonstration of p16<sup>INK4a</sup> and HPV L1 capsid protein as complementary markers can be used to grade precancerous lesions. (Alshenawy, 2014; Balan et al., 2010; Byun et al., 2013; Gatta et al., 2011; Izadi-Mood, Sarmadi, Eftekhari, Jahanteegh, & Sanii, 2014; Yoshida et al., 2008). While L1 reactivity stems mainly from superficial cells where LSIL is present, p16<sup>INK4a</sup> reactivity derives from cells within the entire epithelium, indicating deeper HSIL or SCC lesions.

Dysregulated miRNA profiles have been observed to play a functional role in different human cancers including cervical cancer and may be used as a diagnostic tool. Since most such studies were based on tissue samples, little is known about whether altered expression of these miRNAs can be adequately seen in LBC samples. In this study, we choose *miR-205* as an example to evaluate the possibility of miRNA detection by RT-qPCR method in liquid-based cytology. *miR-205* is considered to be of particular

interest in cxca, where it is often significantly overexpressed compared with normal cells. (Lui, et al., 2007; Witten, et al., 2010; Xie, et al., 2012). *miR-205* has been shown to suppress cell migration/invasion, through the process of epithelial-to-mesenchymal transition, and may serve as an oncogene or tumor suppressor gene, depending on cell type.

Unfortunately, no significant correlation could be found between expression of *miR-205* HPV status and morphology of lesion when analyzing LBC. A slight but not significant trend toward increased expression was found with increasing CIN grade. Here we show that RT-qPCR detection is not ideal for quantification of miRNAs in liquid-based cytology samples using RNAs extracted from the mixed cell population, which contain a large amount of normal cells. As evaluated the cell population under microscopy in the 10 cancer cases, we observed the involved cells of diagnostic interest that are expected to express increased levels of miRNA constitute only a minute proportion of total cell numbers and those cells are often also necrotic and we know that necrosis will affect the quality of RNA molecules.

Theoretically, other single cell based detection method such as in situ hybridization (Ge, Zhang, Liu, Yu, & Chu, 2014; Nielsen, 2012) or microfluidic flow cytometry (Wu, Piccini, Koh, Lam, & Singh, 2013; Wu, Piccini, & Singh, 2014) is a practical method for liquid-based cytology. There is still a long way to put them successfully applicable in clinical usage. To better address this question, more samples specially paired or series samples from same patient are needed.

## 6 8. CONCLUSIONS

The first study shows that LBC better preserves morphology, improves sensitivity for detection of CIN2+ and correlates better with histopathology than conventional Pap smears. LBC testing also allows for ancillary analyses using residual cell suspension.

Performance declined somewhat when this technique was implemented in the population-based screening program. Nevertheless, the new technique achieved screening performance equivalent to conventional cytology. The second study includes the material from this period, which reflects the learning curve from the introduction of the new technology.

Immunoreactivity to p16<sup>INK4a</sup> correlates with the presence of dysplastic lesions and was largely absent in normal cells. Staining indicates oncogenic HPV activity and may therefore find a role in ancillary screening.

Similar reactivity to the HPV L1 capsid protein was associated with mild cellular atypia and was essentially absent in cases of histologically confirmed high-grade lesions. These findings may support the hypothesis that L1 expression is associated with clearing of the infection. Together these two markers may improve identification of precancerous lesions.

The fifth study shows that the LBC technique is inadequate to demonstrate how altered expression levels of tumor-specific *miR-205* reflect the status of established cxa. The low number of tumor cells in LBC samples, as well as the presence of tumor necrosis, make microRNA analysis unsuitable as an indicator for progression to an invasive condition.

## 7 9. FUTURE PERSPECTIVES

Today we know that HR-HPV testing has high sensitivity for the detection of advanced precancerous lesions (Arbyn, et al., 2012; Bulkmand et al., 2007; Ronco et al., 2010). However, the specificity for use as a screening test is limited since most HPV infections are transient and only a small proportion of the screening population has persistent infection associated with risk for progression to malignancy (Koliopoulos et al., 2007; Ronco et al., 2006). Due to the high prevalence of HPV among young women, HPV testing is not recommended as a primary screening tool in younger age groups (Saslow et al., 2012; Saslow et al., 2012). Immunocytochemical demonstration of p16<sup>INK4a</sup> / Ki-67 dual-staining combined with testing for HPV L1 capsid protein reactivity may be helpful when reflex cytology is inconclusive. Along with cell morphology, these three tests may improve identification of women at risk of oncogenic progression. Such use in clinical practice remains to be evaluated.



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